OMEGA-3 FATTY ACIDS IN THE EARLY ORIGINS OF METABOLIC SYNDROME

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ABSTRACT

The role of omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), in particular docosahexaenoic acid (DHA) in decreasing fat deposition, fat cell formation and improving insulin sensitivity *in vitro* and in adult animals had led to suggestions that increasing the supply of these fatty acids before birth could improve later metabolic health outcomes in the child. However, few studies had explored the role of DHA in programming of obesity and type 2 diabetes, and the results had been inconsistent. Furthermore, the mechanisms through which exposure to an increased supply of DHA during development impacted on later health outcomes in the infant were also unclear. While studies in animal models had highlighted the importance of epigenetics in the link between intrauterine nutrition and the subsequent risk of obesity and insulin resistance in the offspring, the role of whether epigenetics in the prenatal programming of obesity in humans was unknown. In addition, while there was evidence that placental alterations played a critical role in developmental programming, few studies had explored the effect of DHA on placental gene expression/function. The central aim of this thesis was to determine the effect of a specific nutritional intervention (maternal DHA supplementation) on (1) markers of metabolic health (BMI, percent body fat, insulin sensitivity) in the children at 5 years of age and (2) global and gene-specific DNA methylation profiles in children at birth and at 5 years of age. I also aimed to determine the effect of DHA on placental proliferation and gene expression *in vitro*.

This thesis studied children born to women who participated in a large randomised controlled trial of n-3 LCPUFA supplementation in pregnancy, the DOMInO trial, at birth and 5 years of age. Insulin sensitivity in these children was estimated from the

HOMA-IR index at 5 years of age. DNA was obtained from blood samples of the DOMInO children collected at birth (n=1012) and at 5 years of age (n=715) for the assessment of global and genome-wide methylation. A human placenta first trimester cell line (HTR8/SVneo) was treated with a DHA-rich emulsion in order to determine effects of DHA on placental proliferation and gene expression.

Maternal DHA supplementation was associated with a reduced insulin sensitivity (increased HOMA-IR index) and increased fasting insulin concentrations in the children at 5 years of age, particularly in males. There were also small but significant differences in methylation of 44 genomic regions at birth and 30 at 5 years of age, and more differentially methylated regions (DMRs) in males compared to females at both time points. DHA treatment increased proliferation rate of HTR8/SVneo cells, and 96 genes were differentially expressed between DHA and no treatment groups. Overall, the results of this thesis provide evidence that maternal DHA supplementation may reduce insulin sensitivity in the children, although whether this translates into differences in the incidence of type 2 diabetes later in life remains to be determined. I also demonstrated that DHA has small but significant effects on DNA methylation of specific genomic regions, and significantly altered proliferation and gene expression of a placental cell line *in vitro*, which suggests that both epigenetic and placental modifications may be involved in mediating the effects of increased DHA exposure before birth on health outcomes in the child.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree of diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this wok will, in the future, be used in a submission for any other degree of diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Jing Zhou

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LIST OF ABBREVIATIONS

5hmeU	5-Hydroxymethalted Uracil
AA	Arachidonic Acid
AID	Activation-Induced Cytidine Deaminase
ALA	Alpha-Linolenic Acid
ANGPTL4	Angiopoietin-Like 4
ANOVA	One-Way Analysis of Variance
AOX	Acyl-Coa Oxidase
APOBEC	Apolipoprotein B Mrna Editing Enzyme
ARHFAP35	Rho Gtpase Activating Protein 35
ASNS	Asparagine Synthetase
BHT	Butylated Hydroxyanisole
BIS	Bioelectrical Impendence Spectroscopy
BMI	Body Mass Index
C	Cytosine
CAST	Calpastatin
CCK	Cholecystokinin
CI	Confidence Interval
CpG	Cytosine Phosphate Guanine
CV	Coefficient of Variation
DHA	Docosahexaenoic Acid
DMAC	Data Management and Analysis Centre
DME	Demeter
DMRs	Differently Methylated Regions
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DOMInO	DHA to Optimise Mother Infant Outcome
DPA	Docosapentaenoic Acid
EPA	Eicosapentaenoic Acid
ESPCR	End-Specific PCR
FA	Fatty Acid
FADS2	Fatty Acid Desaturase
FAME	Fatty Acid Methyl Esters
FDR	False Discovery Rate
FID	Flame Ionisation Detector
FMC	Flinders Medical Centre
FO	Fish Oil
Foligos	Facilitator Oligonucleotides
G	Guanine
GC	Gas Chromatograph
GEE	Generalised Estimating Equation
GR	Glucocorticoid Receptor
HOMA-IR	Homeostatic Model Assessment Of Insulin Resistance

IPA	Ingenuity Pathways Analysis
IQ	Inter-Quartile Range
IQL	Instrument Quantitation Limit
LA	Linoleic Acid
LINE-1	Long Interspersed Nucleotide Element 1
LOD	Limit of Detection
MAGEB3	Melanoma Antigen Family B3
MeCP2	Methyl Cpg Binding Protein-2
MGLL	Monoglyceride Lipase
miRISC	Microrna-Induced Silencing Complexes
MQL	Method Quantitation Limit
mRNA	Messenger RNA
N	Number
n-3 LCPUFA	Omega-3 Long Chain Polyunsaturated Fatty Acids
NFAT5	Nuclear Factor of Activated T-Cells 5
NSCs	Child's Newborn Screening Cards
OECD	Organisation for Economic Co-Operation and Development
PANTHER	Protein Analysis Through Evolutionary Relationships
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate Carboxykinase
POMC	Pro-Opiomelanocortin
PPAR	Peroxisomal Proliferator-Activated Receptor
PPFIBP1	Protein Tyrosine Phosphatase Receptor Type F Polypeptide-Interacting Protein-Binding Protein 1
PTV	Programmed Temperature Vaporization Injector
RBC	Red Blood Cells
RCTs	Randomised Controlled Trials
RISC	RNA-Induced Silencing Complexes
RNA Pol	RNA Polymerase
ROS1	Glycosylases Repressor Of Silencing 1
RQI	Rna Quality Indicator
RSD	Relative Standard Deviation
RXRA	Retinoid X Receptor-A
SAH	S-Adenosyl Homocysteine
SAM	S-Adenosyl Methionine
SAMD4A	Sterile Alpha Motif Domain Containing 4A
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SO	Soy Oil
SREBP	Sterol Regulatory Element Binding Protein
SRM	Serum Reduced Media
STON1	Stonin1
T	Thymine
TDG	Thymine DNA Glycosylase

TLC	Thin Layer Chromatography
VMRs	Variable Methylated Regions
WCH	Women's And Children's Hospital
WHO	World Health Organisation

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

The prevalence of obesity and type 2 diabetes has increased dramatically over the past two decades, and these diseases are now recognised as major public health issues in many countries[1,2].

A large number of epidemiological studies have shown that the environment, in particular the nutritional environment, experienced before birth and in early infancy has a long-term impact on an individual's risk of developing obesity, type 2 diabetes and associated metabolic diseases later in life[3-5]. There is a growing body of evidence which suggests that exposure to either an inappropriately low or inappropriately high nutrient supply before birth and/or excesses or deficiencies of specific dietary components acts to increase the subsequent risk of obesity and type 2 diabetes in the offspring[2,6-8]. This programming of an increased risk of obesity and type 2 diabetes is thought to be due to the ability of nutritional imbalances during development to cause permanent alterations in the expression of genes regulating fat metabolism and energy balance in the offspring[9-12]. However, the mechanisms which underlie this programming of altered gene expression are not clearly understood.

More recently, there has been growing interest in the potential role of omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) in the early programming of fat mass and metabolic health. N-3 LCPUFAs are known to play a critical role in fetal development, particularly the development of the brain and nervous system[13-16]. However, both *in vitro* and *in vivo* studies in adult animals and humans have also shown that the n-3 LCPUFA can reduce fat deposition and improve insulin

sensitivity[17-25]. This has led to suggestions that increasing the supply of n-3 LCPUFA in the maternal diet during pregnancy and/or lactation may protect the offspring against the development of obesity and insulin resistance/type 2 diabetes later in life[26,27]. However, the data from the human and animal studies conducted in this field to date has been inconclusive[26-32]. The clinical trial which provided the source of samples for much of this thesis set out to resolve this controversy. The aim of Chapter 2 was to examine the effect of maternal supplementation with n-3 LCPUFAs, chiefly as docosahexaenoic acid (DHA), in the second half of pregnancy on the risk of developing obesity and type 2 diabetes in the children.

The mechanisms through which an altered nutritional environment in fetal life/early infancy programs an increased risk of obesity and insulin resistance/type 2 diabetes in the offspring, and how n-3 LCPUFA may act to mitigate this risk is also unclear. A substantial body of research in animal models has indicated that epigenetic changes may represent an important link between prenatal nutrition, altered gene expression and heightened risk of obesity and type 2 diabetes in the offspring[12,33,34]. Epigenetic changes are reversible and mitotically heritable DNA modifications that result in altered gene expression and/or alter the way that a gene expression is regulated without a change in DNA sequence[12,33,34]. The most well-described epigenetic modification is DNA methylation which involves the covalent addition of a methyl group to DNA at a cytosine residue[35]. In this thesis, two measurements of DNA methylation were undertaken; global DNA methylation and genome-wide gene/region specific DNA methylation. Global DNA methylation measures the total proportion of cytosine residues across the genome which are methylated vs unmethylated[36,37]; and, genome-wide DNA methylation assesses the degree of DNA

methylation at specific loci across the genome (i.e. gene/region specific DNA methylation)[38].

Animal studies have shown that alterations in the nutritional environment during the perinatal period can affect epigenetic status, including the methylation level, in genes which have an important role in regulating fat deposition and metabolism[35,39-44]. This then might lead to changes in gene expression and gene function which is associated with an altered regulation of energy balance, appetite and/or metabolism and which ultimately leads to the development of obesity and type 2 diabetes[35,39-44]. However, whilst the importance of epigenetic modifications for the programming of metabolic diseases has been demonstrated in animals, human studies that have determined whether nutritional exposures during pregnancy can result in epigenetic alterations at birth or in childhood remain extremely limited. Therefore, the central aim of Chapter 3 and 4 of this thesis was to determine whether maternal n-3 LCPUFA supplementation, chiefly as DHA, in the second half of pregnancy was associated with modifications to either (a) global DNA methylation or (b) genome-wide DNA methylation in the children at birth and/or at 5 years of age. We also aimed to determine whether there was any evidence that global methylation levels at birth or at 5 years of age were correlated with markers of metabolic health, including Body Mass Index (BMI) z-score, percentage body fat and insulin resistance, in the children at 5 years of age.

In addition to effects on the epigenome, studies in both humans and in animal models have suggested that a significant degree of developmental programming is likely to be mediated by effects on the placenta. Previous studies have reported that maternal

dietary restriction, dietary excess, obesity or diabetes results in an altered profile of gene expression in the placenta which impacts on placental function, nutrient transfer and, ultimately, fetal and neonatal outcomes[45,46]. However, despite the large number of studies which have evaluated the effects of maternal DHA supplementation on the child, there is limited knowledge of the effects of DHA on placenta. Therefore, the aim of Chapter 5 of this thesis was to establish, using a placental trophoblast cell line model, the effect of DHA treatment on proliferative activity, apoptosis and gene expression profile of placental cells *in vitro*.

1.2. Obesity and Insulin Resistance/Type 2 Diabetes

1.2.1. Obesity

Obesity is a medical condition which describes an excessive accumulation of body fat that may lead to adverse health consequences[1,8,47]. BMI (weight/height^2) is used to define obesity in adults because it is positively correlated with both total body fat and percentage body fat, at least at a population level[1,8,47]. Individuals with a BMI of more than 30kg/m^2 are classified as obese, while those with a BMI of between 25 and 30kg/m^2 are classified as overweight[2] (Table 1.1). The prevalence of obesity has increased dramatically over the past 3 decades and continues to increase in both developed and developing nations[2]. In 2014, more than 600 million adults (18 years and older) world-wide were obese, and about 1.9 billion adults were overweight[2]. The prevalence of overweight and obesity among Australians has been steadily increasing for the past 30 years[48], and has more than doubled in the past 20 years[48]. In 2011–12, approximately 60% of Australian adults were classified as overweight or obese, and more than 25% of these fell into the obese category[49]. A 2009 report by the Organisation for Economic Co-operation and Development (OECD) predicts that there will be a continued increase in the prevalence overweight and obesity levels across all age groups in Australia, and that these conditions will affect 65% of the population by the end of the next decade[50].

Table 1.1: Categories of BMI[2]

BMI range (kg/m^2)	*Category*
<18.5	Underweight
18.5-25	Normal
25-30	Overweight
>30	Obese

1.2.1.1. Obesity is a Major Health Issue

Obesity has been recognised as a major public health issue across the world since 1997, and is currently the fifth leading cause of global deaths[2]. This is largely because obese individuals have an increased risk of many adverse physical and mental health conditions, including an increased risk of cardiovascular diseases (including ischemic heart disease, hypertension and stroke), type 2 diabetes, musculoskeletal disorders (for example, osteoarthritis), and some types of cancer[2,51]. More than 2.8 million adults worldwide die every year as a result of complications directly associated with being overweight or obese[2]. Epidemiological studies have reported that men who are obese have a significantly higher relative risk of death compared with those who are not, and their relative risk of death increases with increasing BMI[1,47]. As a result, men with a BMI between 30 to 45kg/m^2 have a 2-4 year lower life expectancy and individuals with a BMI of more than 40kg/m^2 have a 10 year lower life expectancy compared to men in the healthy weight range[1,47].

Along with the increasing prevalence of obesity in adults, there has been a dramatic increase in the number of overweight and obese children. In 2007, up to 25% of children aged 2-16 were overweight or obese, with 6% classified as obese world-wide[52]. Obesity in childhood is also associated with adverse health consequences,

including breathing difficulties, increased risk of fractures, hypertension, early markers of cardiovascular disease, insulin resistance and an increased risk of psychological disorders[53]. Importantly, childhood obesity is associated with a higher risk of obesity, premature death and disability in adulthood[53]. Therefore, the prevention of obesity in children is an important strategy for reducing the overall incidence of obesity in the population.

1.2.1.2. Causes of Obesity

Obesity is fundamentally caused by an imbalance between energy consumed and energy expended[1]. It is also clear, however, that the susceptibility to becoming overweight or obese varies significantly between individuals[51,54]. It is therefore important to understand the cause of individual differences in susceptibility to becoming overweight/obese. In this context, it is important that a worldwide series of human and experimental animal studies have shown that an individual's risk of obesity in later life can be influenced by the nutritional environment they experience before birth, and that being exposed to either an insufficient or excessive nutrient supply during early development increases an individual's susceptibility to obesity through the life course[10,12,55-57].

1.2.2. Insulin Resistance and Type 2 Diabetes

1.2.2.1. Insulin Resistance

Insulin resistance is a physiological condition in which cells fail to respond to the normal actions of the hormone insulin, leading to hyperglycaemia. As a consequence of reduced insulin sensitivity in peripheral tissues, the pancreatic beta cells increase their production of insulin, which results in compensatory hyperinsulinemia. The combination of fasting hyperglycaemia and reduced sensitivity to the actions of insulin can progress to type 2 diabetes[58]. A number of factors increase the risk of insulin resistance and type 2 diabetes in humans, including genetic, environmental (physical activity and diet) and pathological (obesity, hypertension or infections) factors[59]. The primary treatment for insulin resistance is dietary modification and increased physical activity, and insulin sensitivity improves significantly in response to weight loss[59]. The gold standard method of measuring insulin resistance is the hyperinsulinemic euglycemic clamp, a measure of the amount of glucose required to maintain euglycemia in the presence of elevated insulin concentration which indicates how sensitive the tissue is to insulin[60,61]. However, since this method is invasive and relatively involved, other methods, such as the modified insulin suppression test, homeostatic model assessment (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI), are often used clinically to provide an estimate of insulin sensitivity.

1.2.2.2. Type 2 Diabetes

Type 2 diabetes mellitus is a metabolic disorder that is characterised by hyperglycaemia, in the context of insulin resistance and relative lack of insulin

secretion[62,63]. Individuals with a fasting venous plasma glucose greater than or equal to 7.0mmol/L or venous plasma glucose greater than or equal to 11.1mmol/L in an oral glucose tolerance test (venous plasma glucose 2 hours after ingestion of 75g oral glucose load) are classified as type 2 diabetic according to the criteria set by the WHO (World Health Organisation) (Table 1.2)[64]. Recent statistics suggest that the number of individuals with type 2 diabetes will double to 300 million globally by 2025 and that it will be 7th leading cause of death by the year 2030[65]. The major risk factor for type 2 diabetes is overweight and obesity and it is possible to improve or reverse this disease with weight loss. Medications such as metformin and the thiazolidnediones are also used to improve insulin sensitivity in individuals where the condition cannot be managed by diet and exercise alone[66].

Table 1.2: 2006 WHO recommendations for the diagnostic criteria for diabetes and intermediate hyperglycaemia[64]

Conditions	***2 hour plasma glucose (mmol/L)***	***Fasting glucose (mmol/L)***
Impaired fasting glucose	<7.8	≥6.1 and ≤6.9
Impaired glucose tolerance	≥7.8 and <11.1	<7.0
Diabetes	≥11.1	≥7.0

1.2.3. Early Life Programming of Obesity and Type 2 Diabetes

It has been well-documented in both human and animal studies that environmental perturbations during prenatal life including changes in nutrient, oxygen and hormone concentrations during critical periods of embryonic and fetal development can lead to lifelong changes in the phenotype of the offspring. Importantly, these changes can result in an increased risk of metabolic diseases, including obesity and type 2 diabetes, later in life[10]. Epidemiological studies in humans, conducted in many different countries, have shown that an individual's birth weight is correlated with their risk of metabolic diseases later in life[3-5,67-75]. A number of experimental animal models have been established to investigate the mechanisms which underlie the association between the prenatal environment and the changes in physiology and metabolism of the offspring[3-5,67-75]. Collectively, these studies have shown that exposure to either an inappropriately low or inappropriately high supply of nutrients or exposure to deficiencies/excesses of specific dietary components during embryonic and fetal development results in altered development of key systems involved in the regulation of energy balance, appetite and fat deposition and persistent alterations in gene expression in the offspring[28].

1.2.3.1. Programming of Obesity and Type 2 diabetes in Humans

One of the earliest and most compelling studies to demonstrate the relationship between birth weight and later metabolic health was a study of 468 individuals who were all born in the same week in Herefordshire, UK. In this cohort, individuals in the lowest quintile of birth weight had an 18-fold higher risk of having the metabolic syndrome (including impaired glucose tolerance, hypertension and hypertriglyceridemia) at 64 years of age compared to those in the highest birth

weight quintile[76]. Since then, a large number of other studies have reported similar associations in populations worldwide[4,5,67,77-79]. Studies of populations in the United Kingdom, North America, India and China have reported that infants who were born either small or large for gestational age have an increased risk of obesity, insulin resistance and type 2 diabetes in child and adulthood compared to average birth weight infants [4,5,10,70-73,80,81]. Thus, there appears to be a U-shaped or J-shaped relationship between weight at birth and the risk of later metabolic disease[6,68,69]. Birth weight has been a useful and convenient proxy marker of the nutritional environment during fetal development in human population based studies, since variations in birth weight in human infants are primarily accounted for by variations in the amount of body fat[7,47,51]. In human infants, body fat usually begins to accumulate from ~25 weeks gestation[3,47]. Hence, lower birth weight and a poorer intrauterine environment, i.e. a reduced substrate supply to the developing fetus, is normally correlated with a lower body fat mass in the newborn[10].

Conversely, there is a growing body of evidence which suggests that exposure to an excess nutrient supply, resulting in an increased birth weight, also acts to increase the risk of obesity and type 2 diabetes in later life[82]. Both maternal obesity and diabetes increase the fat and glucose supply to the fetus, which is associated with increased growth and fat deposition in the infant[3,67,74,82]. Thus, these infants are heavy at birth and tend to stay heavy throughout child and adulthood[3,67,74,82]. These studies have also reported that children born to mothers who were obese or who had diabetes during their pregnancy are at increased risk of developing metabolic diseases, including type 2 diabetes, in child and adulthood[83]. This has led to the creation of an intergenerational cycle of obesity and type 2 diabetes, in which mothers who are

obese or diabetic during their pregnancy are more likely to give birth to infants who go on to develop these diseases later in life[3,67,74,82].

1.2.3.2. Programming of Obesity and Type 2 Diabetes in Animal studies

Whilst there is evidence from human studies that the prenatal nutritional environment is an important determinant of the risk of obesity and type 2 diabetes in the offspring in later life, human studies provide limited insights into the mechanisms which underlie this association. It is also easier to accurately measure the composition and amount of dietary intake in animal studies compared to human studies[5,74,77]. Moreover, the tissues of animals can be accessed to investigate the mechanisms by which prenatal nutrition influences the risk of obesity and type 2 diabetes in offspring.

Models of both prenatal under-nutrition and over-nutrition have been developed in a number of animal species in order to investigate these mechanisms. Two of the most widely used models of under-nutrition include maternal low protein diets and global caloric restriction in rodents[84-90], and both of these dietary treatments are associated with an increased risk of metabolic diseases including increased fat deposition, hypertension, impaired glucose homeostasis and vascular dysfunction in the offspring[10,57,84,87]. The offspring of dams that are fed a globally nutrient restricted diet are growth restricted *in utero*, resulting in a significant reduction in weight and size at birth compared to the offspring of dams fed on standard diets[91]. The offspring of undernourished dams also developed hyperinsulinaemia, hyperphagia, hyperleptinaemia, hypertension, and obesity as adults[91]. Similarly, in guinea pigs, offspring of mothers fed a diet providing 85% of the energy of an *ad libitum* control

diet exhibited altered cholesterol metabolism and lipid homeostasis at 150 days of age[92].

Animal models of prenatal over-nutrition, including maternal high-fat feeding in rodents, sheep and primates and early postnatal over-nutrition in rodents, have shown that exposure to over-nutrition early in life increases the risk of obesity and type 2 diabetes in the offspring through the life course[5,67,71,74,77,80-82]. Interestingly, the impact of maternal over-nutrition on the risk of obesity and insulin resistance in the offspring appear to be independent of effects on birthweight, with different studies reporting either increases[67,82] or decreases [93,94] in birth weight in offspring exposed to maternal over-nutrition during pregnancy.

Thus, both human and animal studies have shown that obesity and insulin resistance/type 2 diabetes can have their origins in early life, and that the quantity and composition of the maternal diet during early development plays an important role in metabolic programming. Experimental animal studies have provided insights into the mechanisms through which this early programming of obesity occurs[10,57,90,95,96], and have consistently demonstrated that exposure to an inappropriate supply of nutrients (i.e. protein, fat or global caloric intake) during development results in persistent changes in gene expression in key tissues and organ systems involved in the regulation of appetite, fat deposition and/or energy metabolism in the offspring[12,56,57,89,97-107]. These changes in gene expression persist after birth, and often throughout the life of the animal, and are associated with changes in the function of key metabolic tissues, including the liver, adipose tissue and appetite regulating neural network[12,56,57,89,97-107]. Whilst the causes of these

persistent changes in gene expression are not known, more recent studies have suggested that they are likely to be associated with epigenetic changes are induced in response to an altered nutritional supply before birth (Figure 1.1)[12,34,39,105,108].

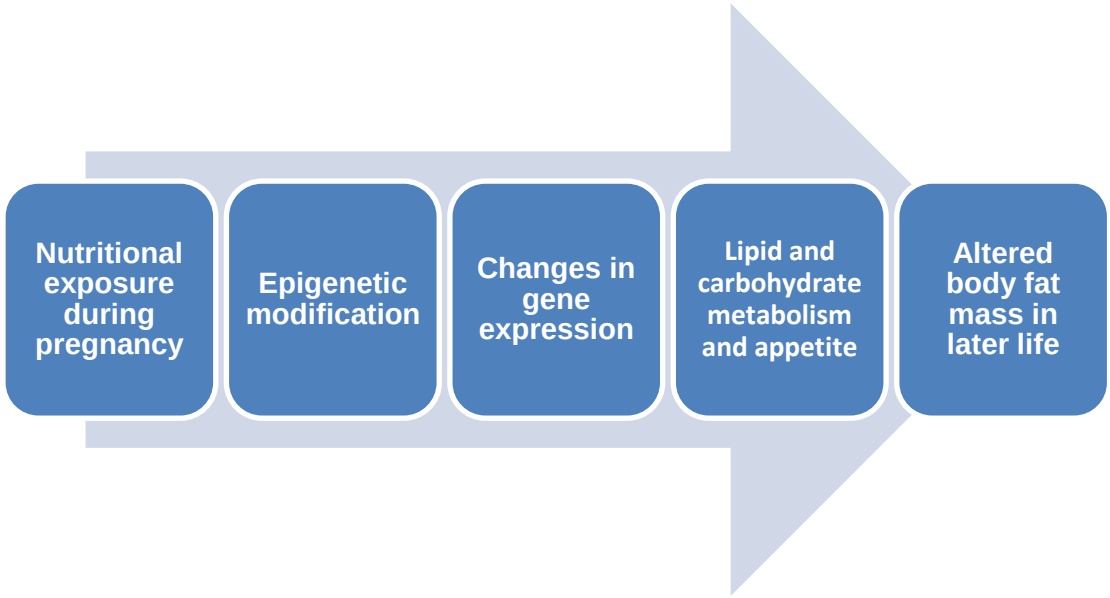

Figure 1.1: Hypothesised pathway linking prenatal nutritional exposures to increased susceptibility to later obesity. According to this hypothesis, specific nutrients in the fetal circulation produce epigenetic modifications in genes related to lipid and carbohydrate metabolism and appetite in the offspring. These epigenetic changes persist after birth, resulting in stable changes in gene expression and thus alterations in the structure and function of these systems. It is also possible that changes in gene expression can contribute to further epigenetic changes. Ultimately, body fat mass and insulin sensitivity in the offspring are persistently altered.

1.3. N-3 LCPUFAs

N-3 LCPUFAs are one of the two major classes of polyunsaturated fatty acids. These fatty acids are characterised structurally by the presence of more than one double bond along the fatty acid chain, with the first double bond located on the third carbon atom from the methyl end[109]. The two most biologically important n-3 LCPUFAs are eicosapentaenoic acid (EPA) and DHA, the structures of which are shown in Figure 1.2[109]. EPA and DHA have been well recognised for their beneficial effects in adults, particularly protection against cardiovascular and inflammatory diseases[15,110,111], and DHA is also highly abundant in nerve tissues and the retina[14]. N-3 LCPUFAs can be obtained preformed from marine sources, including oily fish such as salmon, tuna, mackerel, herrings, sardines and other seafood[109]. They can also be synthesised endogenously from the short-chain precursor, alpha-linolenic acid (ALA), through a series of desaturation and elongation reactions. However, the process of converting ALA through to EPA and DHA is quite inefficient in humans[109]. Hence, an adequate supply of n-3 LCPUFA in the diet is important for ensuring an adequate n-3 LCPUFAs status (blood concentration of these fatty acids). The National Heart Foundation of Australia recommends that Australian adults consume at least 500mg of EPA and DHA every day to assist in maintaining heart health[112].

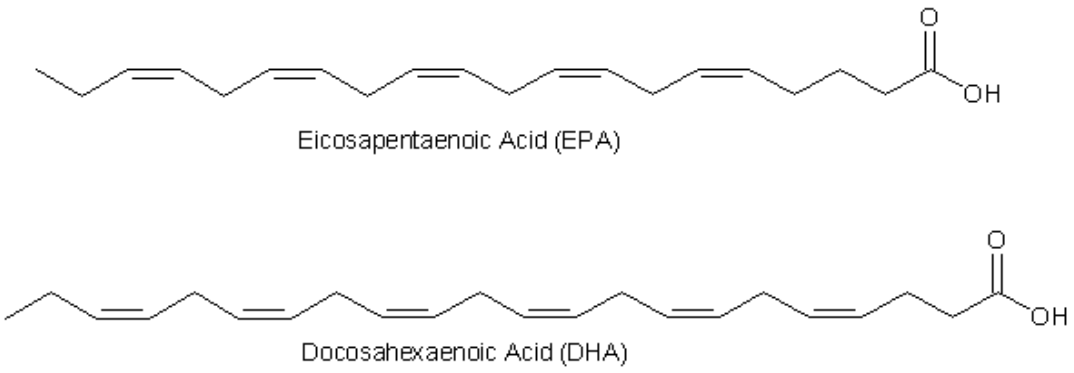

Figure 1.2: Molecular structures of DHA and EPA[109].

1.3.1. N-3 LCPUFAs and the Risk of Obesity and Type 2 Diabetes

Obesity is a metabolic disorder and is associated with increased risk of developing chronic morbidities including hypertension, insulin resistance, type 2 diabetes and dyslipidaemia, which constitute the major components of the metabolic syndrome[17,113]. Recently, there has been growing interest in the potential for increased n-3 LCPUFA intake to have beneficial effects on features of the metabolic syndrome. This is based both on their anti-inflammatory properties and their role in the regulation of lipid metabolism, lipid storage and insulin action[16,109-112].

The excess lipid storage in fat depots in obesity is associated with the infiltration of pro-inflammatory mediators into adipose tissue, and the resulting chronic low-grade inflammation has been identified as a key factor contributing to the development of obesity-related metabolic disorders[114]. EPA and DHA have been shown to alter the functional responses of cells involved in inflammation, resulting in inhibition of: leucocyte chemotaxis and reducing adhesion molecule expression and rleucocyte-endothelial adhesive interaction. In addition, both of the n-3 LCPUFA act to inhibit the, production of pro-inflammatory eicosanoids and cytokines and enhance phagocytosis[115]. These well-established anti-inflammatory properties of n-3 LCPUFA have led to suggestions that increased consumption of EPA and DHA may help to prevent the progression to insulin resistance and type 2 diabetes in overweight/obese individuals[17], [115,116]. In addition to their anti-inflammatory properties, n-3 LCPUFAs have been found to suppress adipogenesis (fat cell formation) and lipogenesis (lipid storage) *in vitro* and in experimental animals and promote fatty acid oxidation in key metabolic organs such as liver, skeletal muscle and white adipose tissue[18-21]. N-3 LCPUFAs mediate these actions by regulating the

activity of key transcription factors including sterol regulatory element binding protein (SREBP) (down regulation) and peroxisome proliferator-activated receptors (PPARs) (up regulation)[117,118].

The physiological effects of n-3 LCPUFA on fat cell formation, fat storage, fat metabolism, insulin sensitivity and inflammation suggest that n-3 LCPUFAs may have a protective role against the development of obesity and its associated metabolic disorders, in particular insulin resistance and type 2 diabetes. However, there is currently limited evidence in support of a relationship between n-3 LCPUFA status and adiposity or insulin sensitivity in humans, with studies in this area to date producing conflicting results[16]. Some studies have supported the suggestion that n-3 LCPUFAs are beneficial for metabolic health. One study, by Micallef and colleagues, for example, reported an inverse relationship between plasma concentrations of n-3 LCPUFAs and BMI, waist circumference and hip circumference[22]. In another study, men with high fish consumption were found to have a reduced risk of overweight; however, no association between n-3 LCPUFAs intake and BMI was shown in a 12 year follow-up study of this same population[23]. In contrast, another prospective cohort study reported that high fish and n-3 LCPUFA intake was associated with an increased prevalence of obesity[24].

In addition to population studies, the potential for increased dietary n-3 LCPUFA intakes to reduce fat accumulation and improve insulin sensitivity has also been explored in a number of randomised controlled trials (RCTs); however these have also yielded inconsistent results. One study which was conducted in 65 overweight adults reported that 12 weeks of supplementation with 0.36g EPA + 1.6g DHA/day

without any other dietary changes resulted in a greater reduction in body fat mass compared to placebo[25]. Similarly, another randomised controlled trial by Kunesova et al. found that supplementing morbidly obese women (n=20) with 1.9g EPA + 0.9g DHA/day for 4 weeks in conjunction with an energy restricted diet (520kcal/day) achieved a significantly greater weight loss compared with the placebo group[119]. Thorsdottir et al. also reported significantly greater decreases in body weight and waist circumference in overweight or obese men who received daily fish or fish oil supplementation in conjunction with a very low energy diet (600kcal/day) for 8 weeks compared with the placebo group[120]. Another study also showed that fish oil supplementation, together with a reduced calorie diet, was associated with greater decreases in body fat, fat mass, and adipocyte diameter of subcutaneous tissue in healthy adults or women with type 2 diabetes, than achieved with the low calorie diet alone[121,122].

Despite these positive results, a number of other RCTs have failed to demonstrate any beneficial effects of n-3 LCPUFAs supplementation on body weight or body fat loss. DeFina et al. reported that 6 month period of n-3 LCPUFA (EPA: DHA=5:1, 5g/day) supplementation had no additional effect on body weight reduction, body fat loss or waist circumference compared to dietary restriction alone[123]. In another study, obese adults who received 14 weeks of n-3 LCPUFA supplementation (70mg EPA and 270mg DHA/day) together with an energy-restricted diet, exhibited similar weight loss and improvements in metabolic profile compared with the placebo group[124]. Similarly, another study reported that overweight or obese insulin-resistant women who received fish oil supplementation (1.3g EPA+ 2.9g DHA/day) together with an energy restricted diet had the same body weight after 5 weeks of treatment

compared with the placebo control group[125]. Thus, the potential for n-3 LCPUFA supplementation to improve metabolic health in humans remains controversial.

1.3.2. Roles of n-3 LCPUFAs in Perinatal Development

The n-3 LCPUFAs, in particular DHA, play a particularly important role in fetal and neonatal development in humans, especially the development of the visual, brain and nervous system[126-128]. This has led to numerous clinical studies that have focussed on determining whether exposure to an increased supply of DHA before birth and in early infancy has beneficial effects on the cognitive, visual and behavioural development[129,130]. Some epidemiological studies have reported that higher maternal intake of fish during pregnancy is associated with improved cognitive function in their children aged from 6 month to 8 years[131,132]. Some of the RCTs which have been conducted in this area have also demonstrated positive effects of maternal n-3 LCPUFA supplementation on neurodevelopmental outcomes in children[133-135]. However, a meta-analysis of RCTs of maternal fish oil supplementation concluded that there was insufficient evidence of a beneficial effect of maternal fish oil supplementation on cognitive outcomes in children, despite positive results in some smaller studies[136]. This is supported by recent results from the largest double-blind RCTs of DHA supplementation in pregnancy conducted to date, the DHA to Optimise Mother Infant Outcome (DOMInO) trial, which also found no significant beneficial effect of maternal DHA supplementation on cognitive composite scores and mean language composite scores in their children, either at 18 months or 4 years, when compared with the placebo group [128,137]. Thus, there is currently no robust evidence for a beneficial effect of maternal n-3 LCPUFA supplementation during pregnancy on cognitive outcomes in children.

1.3.3. N-3 LCPUFAs and Programming of Obesity and Type 2 Diabetes

The role of n-3 LCPUFA in decreasing fat deposition, fat cell formation and improving insulin sensitivity *in vitro* and in adult animals had led to suggestions that an increased supply of these fatty acids during fetal life and early infancy could improve later metabolic health outcomes in the child[26,28,29,138]. However, there are currently few studies which have explored the role of n-3 LCPUFAs in programming of obesity and type 2 diabetes. One study which examined the relationship between maternal circulating DHA level and fetal insulin sensitivity by measuring cord blood glucose and insulin concentrations, concluded that low circulating DHA levels were associated with compromised fetal insulin sensitivity, and may therefore be involved in prenatally "programming" the susceptibility to type 2 diabetes in the offspring of mothers with gestational diabetes[13]. Some studies in rodents have also shown that increased n-3 LCPUFAs in the diet during pregnancy and lactation decreased weight and blood pressure in the adult offspring[139], and it has therefore been suggested that n-3 LCPUFAs supplementation during pregnancy could potentially decrease the risk of obesity and improve insulin sensitivity and cardiometabolic health in the offspring. Although the results of animal studies have generally been positive, closer examination of the studies reveals that only two have actually confined the n-3 LCPUFA supplementation to the prenatal and suckling periods, and one of these two studies reported that fat mass was actually increased in the offspring of DHA-supplemented dams at 6 weeks of age[139].

The effect of maternal supplementation with n-3 LCPUFAs during pregnancy and/or lactation on BMI of the children has been examined in only a small number of human trials[26,29,30,140], and these have produced conflicting results. Donahue and

colleagues reported that an enhanced maternal n-3 LCPUFA status was associated with lower childhood adiposity[141]. One study reported that the weight and BMI of infants born to mothers supplemented with n-3 LCPUFAs (200mg DHA/day) during pregnancy and lactation was significantly lower compared to the control group at 21 months of age[26]. A follow-up of the children from this trial, however, reported that no effect of the n-3 LCPUFA supplementation on BMI at 6 years of age[138]. Interestingly, another study found that the children of mothers given fish oil during pregnancy had a significantly higher BMI at 2.5 years of age compared to children of mothers in the placebo group[30], while a third study found that n-3 LCPUFA supplementation during the lactation period had no effect on BMI in the children at 7 years of age[29]. Hence, the effects of n-3 LCPUFA supplementation during fetal development on the risk of obesity in offspring later in life remain unclear, and more studies are needed to investigate whether n-3 LCPUFA supplementation in humans could reduce the risk of obesity and insulin resistance/type 2 diabetes in infants and children. No human studies have determined the effect of increased prenatal n-3 LCPUFA on insulin sensitivity in the children, and this remains an important area for future research.

1.4. Epigenetic Modifications

Epigenetic modifications are defined as alterations to DNA which result in the alteration of gene expression without a change in the DNA sequence and may persist through cycles of cell division[33]. Epigenetic processes are particularly important for cellular differentiation during early development, in which a single zygote with a single cell type develops into an embryo and fetus which contains many different cell lineages such as muscle cells, blood vessels cells and neurons[33]. Even though each different type of cell has the same DNA sequence, they have different profiles of gene expression and different properties because each cell type carries different epigenetic modifications (sometimes called the epigenome) and thus a different set of genes are expressed[10].

In addition to their role in normal development, there is growing evidence that epigenetic processes also play an important role in the developmental origins of various diseases including allergic and metabolic diseases[142]. For example, animal studies have shown that supplementing the maternal diet with methyl donors increased allergic inflammation in the offspring by increasing global and gene-specific DNA methylation[142].

1.4.1. DNA Methylation

DNA methylation is currently the best characterised of the epigenetic modifications, and changes in DNA methylation play a critical role in the development of organisms[33]. DNA methylation involves the attachment of a methyl group (CH_3) at the number 6 nitrogen of the adenine purine ring or the 5 position of the cytosine pyrimidine ring[33] (Figure 1.3). In humans, DNA methylation mainly occurs on the position 5 carbon of CpG dinucleotides, which are cytosine and guanine nucleotides linked by a phosphate group in the DNA chain[33].

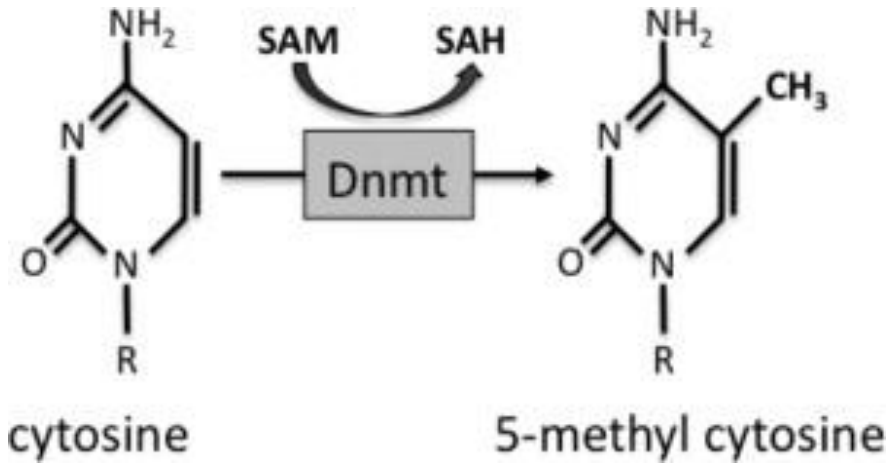

Figure 1.3: The most common methylation process is attachment of a CH_4 at the 5 position carbon in cytosine pyrimidine ring by DNA methyltransferase (DNMT)[33]. The methyl group (CH_3) is contributed from the co-substrate S-adenosyl methionine (SAM) which is converted to S-adenosyl homocysteine (SAH) during the methylation process[142].

Early development is a time during which there are particularly dynamic changes in the methylation status of genes[10]. In early pregnancy the majority of genes in the zygote are unmethylated, due to a process in the genome initiated after fertilisation in which 60% to 90% of CpG dinucleotides become de-methylated[10]. The genes of the

zygote are then re-methylated in a step-wise fashion during the subsequent stages of fetal development (Figure 1.4)[10]. Even though the reason for demethylation/re-methylation process still is not completely understood, studies have shown that DNA methylation is important for cellular differentiation and organ development in humans[10], and that it can change the pattern of gene expression in the cells or maintain gene silencing[41].

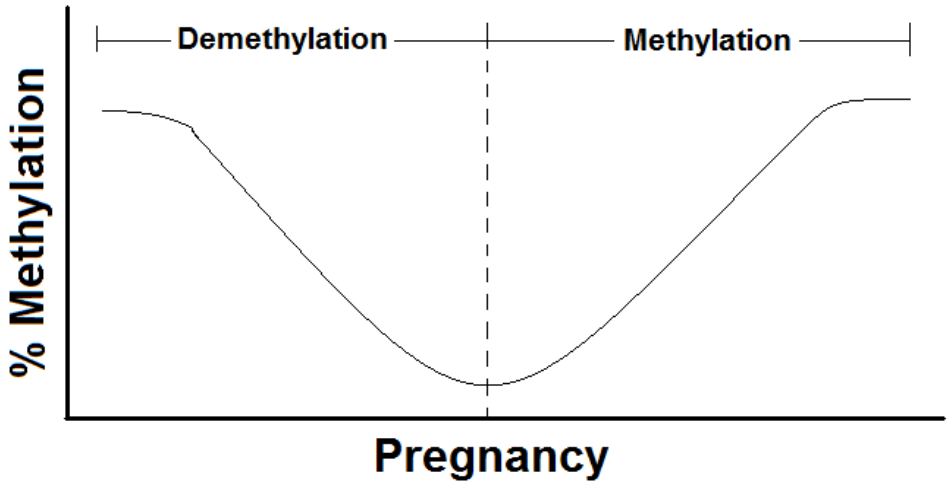

Figure 1.4: After fertilisation, the embryo's genome undergoes almost complete demethylation. Then, just prior to implantation, the genome undergoes step-wise re-methylation in a gene-specific manner[10].

There are two ways that DNA methylation influences gene transcription[10]. The first is the methyl group preventing binding of transcription factors to the promoter region of the gene, thereby blocking its transcription[10]. The second is by the methylated CpG recruiting the methyl CpG binding protein-2 (MeCP2) to the coding region (Figure 1.5)[10]. This MeCP2 recruits other histone-modifying complexes resulting in a closed chromatin structure and transcriptional silencing[10]. The binding of other

complexes can also remodel the structure of the chromosome which permanently inhibits the transcription of the affected gene. Both of these pathways suppress DNA transcription[10].

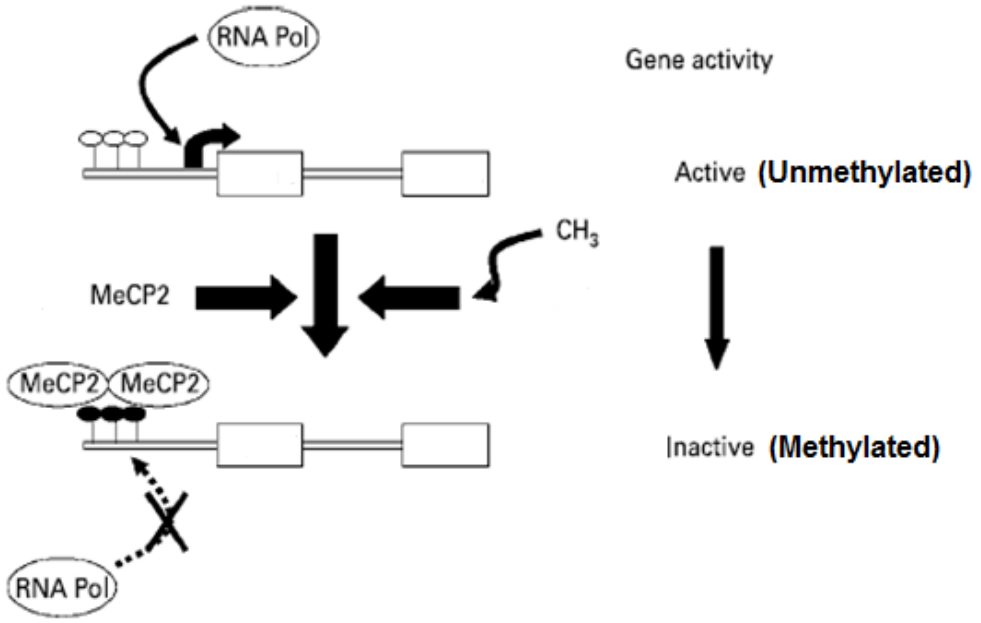

Figure 1.5: RNA polymerase (RNA Pol) binds the coding region when it is unmethylated. MeCP2 binds to the coding region when it is methylated. CH_3 denotes the methyl group[10].

1.4.1.1. Assessments of DNA Methylation

DNA methylation can be assessed as both global DNA methylation and gene-specific CpG island methylation. Global DNA methylation refers to the overall level of 5-methylcytosine in genome[37] and can be measured by using repeat interspersed regions such as *Alu* or Long Interspersed Nucleotide Element 1 (LINE-1). LINE-1 is the most abundant family of non-long terminal repeat retrotransposons found in the genome, making up about 17% of the human genome[143,144]. Such elements have

served as a useful proxy for global DNA methylation as they are commonly heavily methylated in normal tissues, and are spread ubiquitously throughout the genome[145]. Most of the methods used to measure DNA methylation are based on the ability of sodium bisulfite to selectively deaminate cytosine, but not 5-methylcytosine, to uracil[142,143]. Thus, treating DNA with sodium bisulfite allows the researcher to distinguish cytosine from 5-methylcytosine[146,147]. After the bisulfite conversion, the sequence differences between a methylated and unmethylated cytosine can be exploited by either direct sequencing, restriction digestion[148], nucleotide extension assays[149], primer-specific PCR[150] or pyrosequencing[147,148]. Other than the bisulfite conversion techniques, end-specific PCR using a methylation-sensitive restriction enzyme can also be used as a sensitive measure of global DNA methylation[36].

Gene-specific CpG island methylation assesses the methylation status at a specific locus or region which can be associated with gene regulation, as described in the previous paragraphs[38]. Genome-wide DNA methylation analysis provides the profile of locus-specific methylation status across the whole genome[38].

1.4.2. Histone Acetylation

Histone acetylation regulates the transcription of numerous genes[151]. This process involves the addition of an acetyl group on the N-terminal tail of lysine residues under the catalysis of histone acetyltransferase[151] (Figure 1.6). Histone acetylation gives rise to a negative charge on lysine residues to neutralise the positive charge of the histone[151]. This neutralisation decreases the interaction between phosphate groups of DNA, which also have a negative charge, and the N-terminal of the histone and results in a more relaxed structure in the condensed chromatin[151]. This makes the DNA more accessible to transcriptional machinery and therefore results in enhanced transcription of the gene[151]. Conversely, histone acetylation can be reversed by histone deacetylases through histone deacetylation, and this leads to a tighter configuration of the condensed chromatin and reduced expression of the gene[151,152].

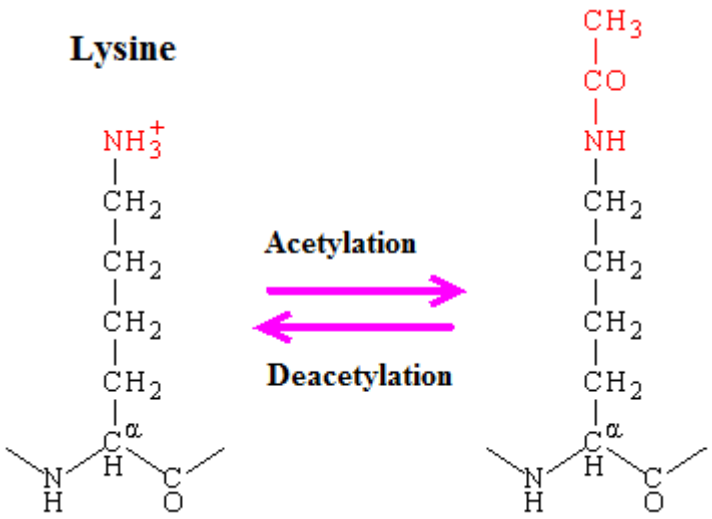

Figure 1.6: The process of acetylation and deacetylation on the lysine residue[153].

1.4.3. MicroRNA

MicroRNAs are small single-stranded noncoding RNAs with a length of around 22 nucleotides[154]. They exist in most eukaryotic organisms to mediate gene silencing post-transcription by inhibiting of translating mRNA into protein or destabilising mRNA[154]. They are abundant in many human cells; the human genome is thought to encode over 1000 microRNAs, which target around 60% of mammalian genes. microRNAs are involved in the variety of processes such as development, cell proliferation, differentiation and apoptosis. They regulate numerous metabolic processes including insulin secretion, heart muscle development, hematopoietic lineage differentiation, apoptosis, and neuron development[155-157].

MicroRNAs are processed from primary transcripts in two main steps: transcription and cleavage (Figure 1.7)[154]. Either discreet miRNA genes or introns of protein coding genes are transcribed to pri-microRNA by RNA Pol II/III in nucleus[154]. Pri-microRNA is then cleaved into pre-microRNA (which has around a 70nt hairpin structure) by RNase III-type enzyme, Drosha- DiGeorge syndrome critical region 8(Drosha-DGCR8)[154]. Once pre-microRNAs are transported into the cytoplasm by Exportin-5-RanGTP[158], they are processed into double stranded RNAs (approximately 22bp length) by the RISC loading complex, which comprised a RNase Dicer, binding protein TRBP, protein activator of PKR and Argonaute proteins[154]. The mature microRNA (one of two strands of processed microRNA) is loaded onto the Argonaute proteins to form microRNA-induced silencing complexes (miRISC)[154], which act to inhibit target messenger RNA (mRNA) translation by reacting with their complementary sequences[154].

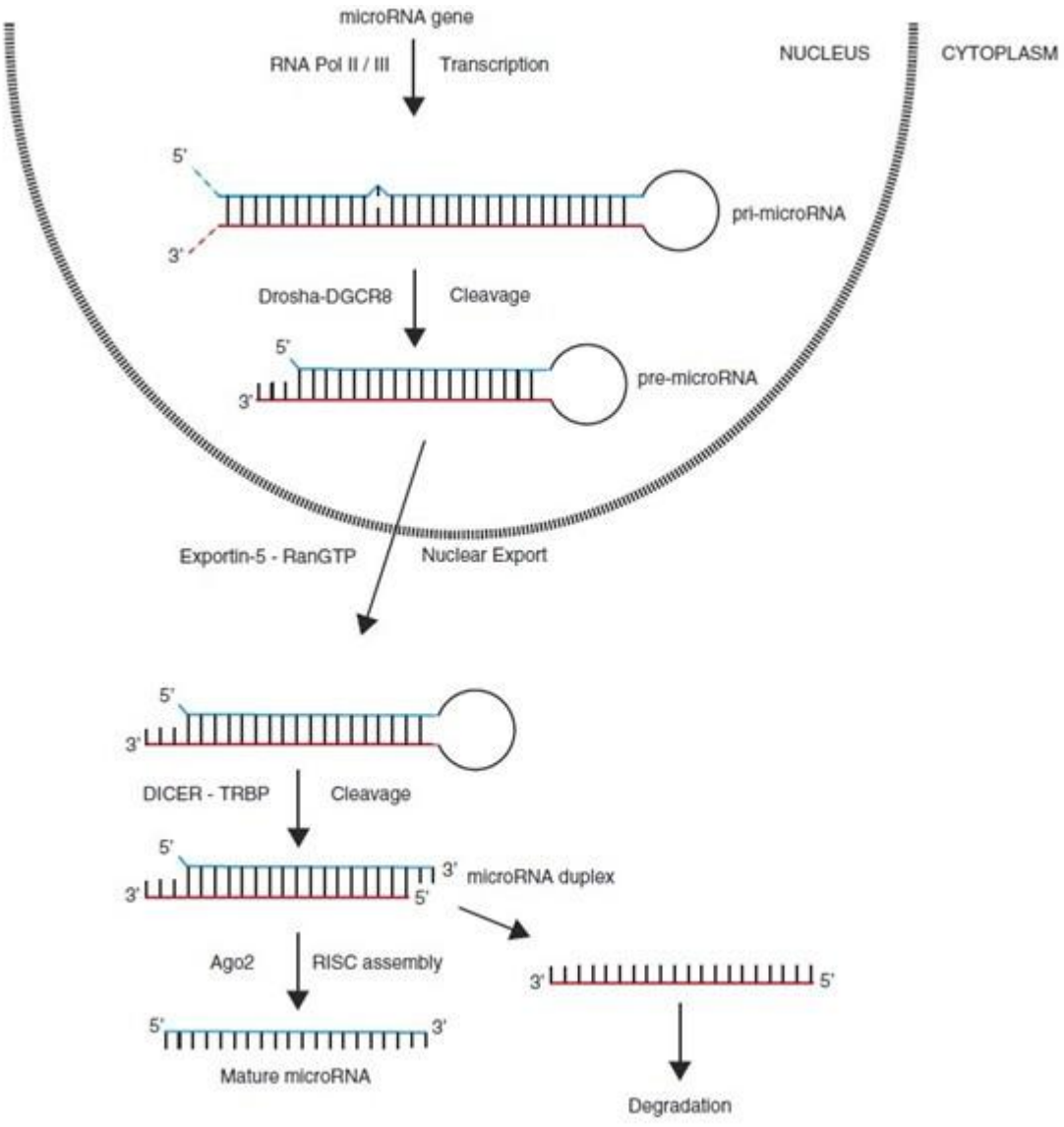

Figure 1.7: Mechanism of microRNA biogenesis. The nuclear membrane is presented as a broken curved black line.

In conclusion, epigenetic modifications are critical for gene expression and cellular differentiation during development, and the long-term stability of some epigenetic marks can be associated with latent effects on disease predisposition. If the embryo or fetus is exposed to an environmental perturbation during the re-methylation of the genome, or during other sensitive developmental windows, then this could

potentially interfere with the normal process of re-methylation and thus lead to permanent changes in gene expression or the regulation of transcription of a particular gene or set of genes[10]. If this gene or genes codes for a peptide related to lipid metabolism, energy balance regulation or insulin signalling, then these epigenetic changes have the potential to cause long term changes in the structure and function of systems related to fat accumulation and insulin sensitivity or glucose tolerance and thus impact on an individual's subsequent risk of obesity and type 2 diabetes[10]. Hence, the impact of the nutritional environment experienced during early development on epigenetic status of the newborn provides a mechanism through which the function of key metabolic genes, and hence the risk of obesity in offspring, can be influenced by prenatal nutrition.

1.5. Prenatal Nutrition and Epigenetic Changes in the Offspring

1.5.1. Animal Models

Although the role of epigenetics in the early life origins of obesity and type 2 diabetes in humans is not completely understood; there is considerable evidence from animal studies that epigenetic process have an important role in the mechanistic pathway through which the prenatal nutritional environment impacts on the susceptibility of the offspring to obesity and type 2 diabetes in later life.

Altering maternal nutrient intake has been shown to affect the DNA methylation status of specific genes which regulate lipid and carbohydrate metabolism and which are involved in the central regulation of appetite, and thereby lead to permanent alterations in the structure and function of these systems in the offspring[10,12,96]. Table 1.3 presents a summary of existing animal (predominately rodent) studies which have investigated the association between maternal nutrition in pregnancy and epigenetic changes in specific genes involved in vascular, endocrine or metabolic function. All of these epigenetic changes have been assessed by measuring DNA methylation status of candidate genes in individual target tissues or in blood.

Metastable epialleles, alleles that are variably expressed in genetically identical individuals due to epigenetic modifications that were established during early development, have been particularly valuable for studying the impact of environmental exposures in utero on the offspring epigemone[159-162]. One model for such epigenetically based phenotypic variability is the viable yellow agouti (A^{vy}) mouse[159].The viable yellow agouti (A^{vy}) allele, whose expression is correlated to DNA methylation, is a murine metastable epiallele, which has been used as an

epigenetic biosensor for environmental factors affecting the fetal epigenome[163].. This is because the expression of the Agouti gene is known to be epigenetically regulated and codes for a phenotype which can be readily identified[164] When the Agouti locus is methylated, the gene is silenced and the coat colour of Agouti mice is brown. Conversely, when the Agouti locus is unmethylated, the gene is expressed and the coat is yellow[164]. Hence, the DNA methylation status of the Agouti gene in any individual mouse can be easily identified from its coat colour. In Agouti mice, supplementation of the maternal diet during pregnancy with methyl donors, including betaine, choline, folic acid and vitamin B, has been shown to result in an increase in the proportion of offspring born with brown coats, indicating that the higher availability of methyl groups in the maternal diet results in increased methylation at the Agouti locus in the offspring[164]. These results provided the first evidence that the maternal diet during pregnancy could alter epigenetic marks in the offspring and result in stable changes in gene expression, and therefore phenotype, of their offspring in postnatal life[164]. Moreover, these studies showed that these epigenetic changes which occurred *in utero* could have effects on the phenotype of offspring which persisted for their entire life[10,164].

Animal models of under-nutrition, including maternal low protein diets and global under-nutrition during pregnancy have provided evidence that these nutritional exposures can also alter the epigenetic status of important metabolic genes (Table 1.2). A series of studies by Burdge and Lillycrop[95,96,102,103,165] found that maternal protein restriction was associated with decreased DNA methylation of the glucocorticoid receptor (*GR*) and *PPAR-α* promoters[12,95,96,98,102,103] and increased mRNA expression of these genes in the liver of the offspring[10,12,96]. Both GR and

PPAR-α play a central role in regulating hepatic gluconeogenesis and lipid metabolism, and the up-regulation of these genes was associated with significant impairments in liver function in protein-restricted offspring[96,104,164]. This suggested, therefore, that alteration of epigenetic regulation of genes during development could influence the transcription of specific genes and their related metabolic functions, including lipid metabolism and fatty acid storage, in offspring organs through the life course[10]. Conversely, maternal under-nutrition induced through caloric restriction in the dams during pregnancy has been reported to result in increased methylation of the *GR* and *PPAR-α* promoters, thereby decreasing the expression of these genes, again providing a mechanism underlying the altered liver metabolism and susceptibility to insulin resistance, glucose intolerance, dyslipidemia and increased fat mass in these animals[91]. Both sets of studies provide evidence that low protein diets and global under-nutrition during pregnancy can influence metabolic function in the offspring by altering the level of DNA methylation in specific target genes, leading to long-lasting changes in gene expression and associated metabolic dysfunction. These epigenetic events therefore appear to have a central role in the pathway linking altered prenatal nutrition with poor metabolic outcomes after birth. Importantly, these epigenetic changes sometimes can be transgenerationally inherited, such that nutritional perturbations in the F0 generation can be stably transmitted to the F2 progeny[33,35].

There have been fewer studies investigating the role of prenatal over-nutrition in early development on epigenetic regulation of genes related to metabolic phenotypes including obesity, hyperleptinaemia, hyperglycaemia and hyperinsulinaemia in the offspring[107]. However, Plagemann and colleagues showed that rats exposed to early

postnatal over-nutrition induced by reducing litter size to 3 pups at birth, had increased DNA methylation of two CpG dinucleotides within the promoter region of the appetite-inhibiting neuropeptide, pro-opiomelanocortin (*POMC*) promoter after weaning[107]. This suggested that the decrease in *POMC* expression reported in earlier studies by this group was due to altered methylation of this gene, and the lower expression of this appetite inhibitor would also account for the increased appetite and susceptibility to weight gain seen in these animals[107]. Hence, this study indicated that over-nutrition during pregnancy could increase the susceptibility to obesity in later life via a persistent alteration in the methylation level of a gene which regulated energy homeostasis (Figure 1.8).

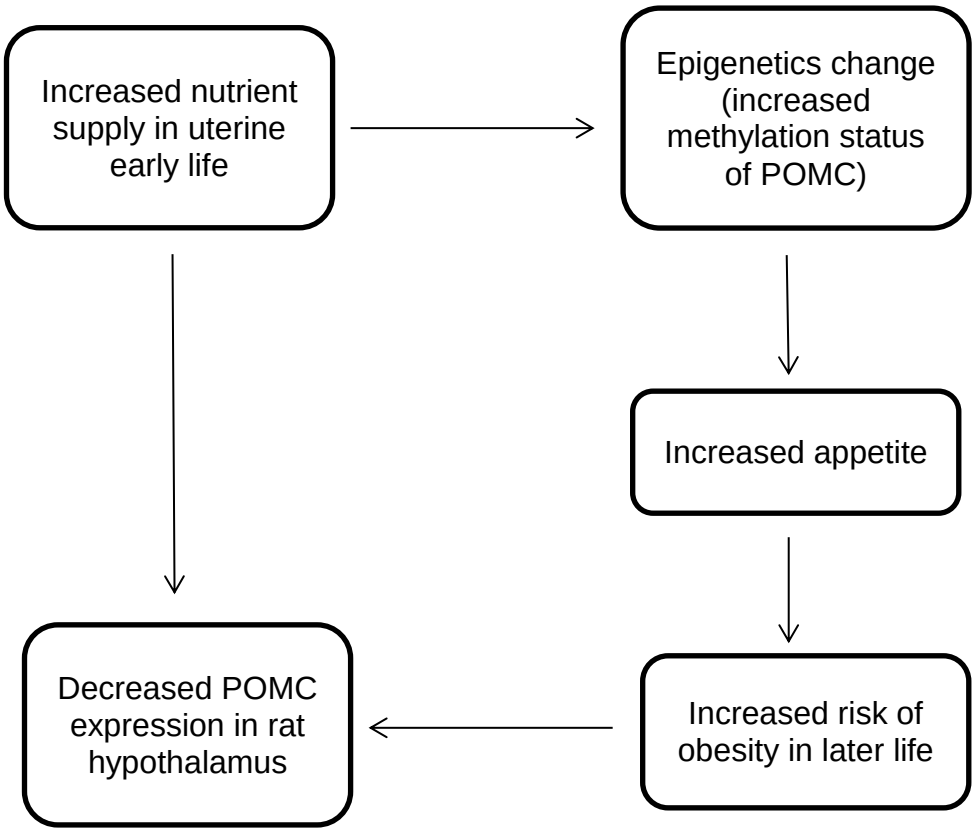

Figure 1.8: Schematic representation of results of study by Plagmann and colleagues in relation to effects of early life over-nutrition on POMC methylation and associated phenotype (increased appetite and increased fat deposition) of offspring post-weaning[107].

Table 1.3: Summary of studies which have investigated association between nutritional intake of dams during pregnancy and altered methylation level in specific genes, related to metabolic function

Reference	*Species*	*Intervention*	*Intervention Period*	*Age of Offspring at Time of Assessment*	*Assessed Genes*	*Methylation Change in Intervention Group*
Bertram C, et al. 2001[98]	Rat	18% casein (control) or 9% casein	Pregnancy	4, 8, 12, and 16 weeks of age	GR	↓
					PPAR-α	↓
Lillycrop KA, et al. 2005[95]	Rat	180g protein/kg or 90g casein/kg	Pregnancy	6 days after weaning	GR	↓
					PPAR-α	↓
					PPAR-γ1	Unchanged
Lillycrop KA, et al. 2007[96]	Rat	180g casein/kg or 90g casein/kg	Pregnancy and lactation	34 days after birth	GR	↓
					PPAR-α	↓
Burdge GC, et al. 2006[102]	Rat	18% (w/w) (control) or 9% (w/w) casein	Pregnancy	80 days of age	GR	↓
					PPAR-α	↓
Burdge GC, et al. 2004[103]	Rat	18% (w/w) (control) or 9% (w/w) casein	Pregnancy	6 days after weaning	PPAR-α	↓
					PPAR-γ1	Unhchanged
Waterland RA, et al. 2003[164]	Mouse	Methyl donors and cofactors folic acid, vitamin B_{12}, choline chloride and anhydrous betaine	Pregnancy and lactation	21 days after weaning	Agouti gene	↑
Gluckman PD, et al. 2007[91]	Rat	Under-nutrition 30% of *ad libitum*	Pregnancy	170 days of age	GR	↑
					PPAR-α	↑
Burdge GC, et al. 2009[165]	Rat	18% (w/w) or 9% (w/w) casein	Pregnancy	84 days of age	GR	↓
					PPAR-α	↓
Plagemann A, et al. 2009[107]	Rat	Small litter size: 3 litters/nest Normal: 12 litters/nest	Lactation	Postnatal day 21	POMC	↑

1.5.2. Human Studies

Although there is increasing evidence that epigenetics has an important role in the early origins of obesity in animal studies, this has not been clearly demonstrated in humans. A recent paper from Godfrey and colleagues, however, showed that decreased intake of carbohydrate during pregnancy was associated with increased DNA methylation of the retinoid X receptor-α (*RXRA*) in the umbilical cord blood at delivery[105]. The increased methylation status of this gene was also associated with increased body fat mass in the children at 9 years of age[105]. However, this was an observational study and doesn't demonstrate clearly that there is a cause and effect relationship between maternal nutrition and epigenetic status in infants/children. Another study conducted in the Dutch Hunger Winter cohort[166], suggested that individuals who were exposed to famine during early gestation had reduced DNA methylation of the imprinted insulin-like growth factor II (*IGF2*) gene in adulthood compared with their unexposed, same-sex siblings[167]. This study suggested that the early life nutritional environment could induce life-long epigenetic changes in humans[166]. More direct evidence for the involvement of epigenetics in the response to altered perinatal nutrition comes from a recent randomised controlled trial. This study reported that maternal n-3 LCPUFA supplementation (400mg/day DHA) during pregnancy increased global DNA methylation levels compared to control group (olive oil) in cord blood of children (n=52) whose mothers were smokers[32], providing the first evidence that increasing the intake of a specific nutrient (DHA) during fetal development in addition to global under-nutrition has the potential to result in altered DHA methylation in the children at birth[32]. Thus, while very few human studies have been conducted to date, the results of those published thus far have supported the suggestion that the nutritional environment during embryonic and

fetal development, including global nutrition and specific nutrients (including n-3 LCPUFA), can cause epigenetic modifications in human infants and children, and that these may therefore contribute to the early origins of obesity and metabolic diseases in human populations induced by altered prenatal nutrition.

1.6. The Placenta

Fetal growth is largely dependent on the availability of nutrients to the fetus during development[46]. Failure to grow normally *in utero* is associated with an increased risk of complications at birth as well as increased mortality and long-term morbidity[46]. As the only organ connecting mother and fetus, the placenta is the single most important organ for fetal development and provides oxygen, water, carbohydrates, amino acids, lipids, vitamins, minerals and other nutrients to the fetus from the maternal circulation, whilst removing carbon dioxide and other waste products[46,168]. It also metabolises a number of substances and can release metabolic products into the maternal and/or fetal blood[46,168]. The placenta also releases hormones into both the maternal and fetal circulations which fulfil a wide range of functions, including contributing to the regulation of maternal and fetal metabolism, fetal growth, and the initiation of parturition[46,168]. In addition, the placenta also acts as a selective barrier for substances, including xenobiotics and certain toxins, that could be circulating in maternal blood and restricts their movement into the fetal circulation[46].

The human placenta is a haemochorial villous organ, in which maternal blood comes into direct contact with placental trophoblast cells and allows an intimate relationship between the developing embryo and its supply of nutrients[45]. Placental formation and function is a highly coordinated process involving interactions between maternal and embryonic cells[45,46]. The invasive extravillous trophoblasts of the human placenta are critical for successful pregnancy outcome [169]. Trophoblast cell invasion of uterine tissues and remodelling of uterine spiral arterial walls ensures that the developing feto-placental unit receives the necessary supply of blood and that efficient transfer of nutrients and gases and the removal of wastes can take place[169-171]. Dysfunction of

extravillous trophoblasts invasion is directly involved in pre-eclampsia which is a major and frequent complication of human pregnancy with serious fetal and maternal consequences[172].

The crucial role that the placenta plays in fetal development means that changes or defects in placental development or function have significant effects on the development of the fetus and, consequently, on long term health outcomes[46]. The placenta acts as an important mediator of the effect of altered maternal nutrition during pregnancy on the development of the fetus, and therefore plays a critical role in developmental programming[45]. A large number of studies have explored the association between placental growth and functional capacity, mainly determined by its weight and volume, and the incidence of obesity and metabolic diseases in the offspring[45]. A number of studies have demonstrated an association between placental size or fetal/placental weight ratio and the risk of metabolic diseases in the offspring[45,173-177]. However, measuring the weight and volume of placenta accurately is difficult, and can be affected by blood and other factors[45]. It is therefore still unclear whether the weight or volume of the full-term placenta is related to an individual's future risk of metabolic diseases in humans[45].

Placental function is influenced by a wide range of maternal factors, as well as factors released by the placenta itself[168,178]. As shown in Figure1.9, maternal factors including diet, exercise, body composition, metabolism and endocrinology affect the function of the placenta both in terms of its nutrient transport capacity and endocrinology (i.e. the profile of hormones secreted into the maternal and fetal circulations)[46,178]. Changes in the maternal environment such as changes in nutrient

intake can also affect placental structure, which in turn alters both function and fetal vascular resistance[45,46]. Changes in placental function can cause redistribution of fetal blood flow and changes in the regulation of gene expression, which may affect the normal pattern of development[46], and the alteration in gene expression and organ development may persist into adult life and have long-term effects on health[46]. Hence, more studies are needed to examine the impact of different nutritional exposures on the function and/or gene expression prolife of the placenta.

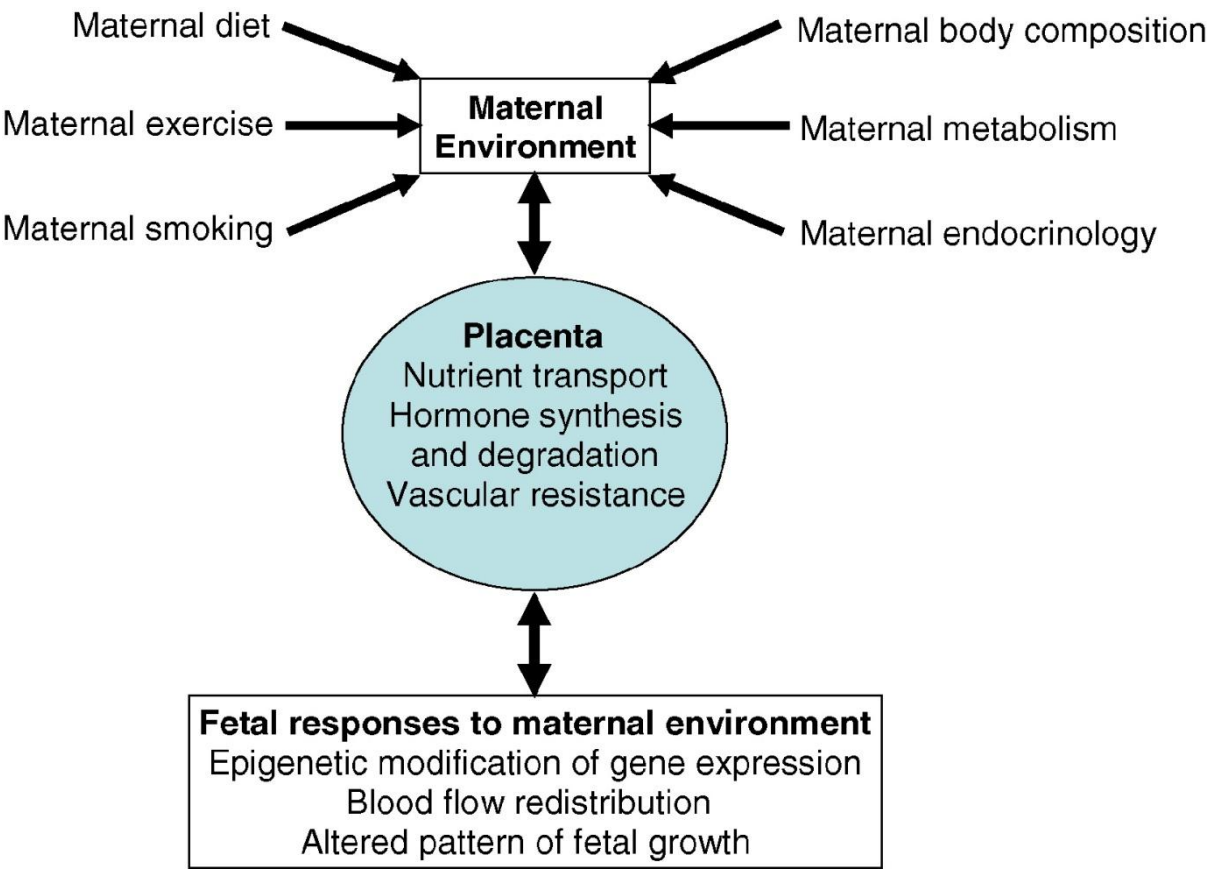

Figure 1.9: The placenta mediates the effects of the maternal environment on the fetus[46].

1.6.1. The Placenta as a Mediator of Developmental Programming

The placenta is the central organ responsible for nutrients transferring to the fetus[168], and alterations to its structure and/or function has been shown to make a significant contribution to developmental programming[45]. Epidemiological studies have identified associations between placental weight, placental volume and placental weight/birth weight ratio and a range of metabolic outcomes, including blood pressure, coronary heart disease, stroke and glucose tolerance in adulthood [45,168,178]. One study conducted in the UK and including 2,571 men, showed a U-shaped relationship between placental weight/birth weight ratio and the incidence of coronary heart disease in adulthood[175]. Thus, both a low and a high placental weight/birth weight ratio were associated with an increased odds ratio of death from coronary heart disease [175]. A study of 3,639 men and 3,447 women conducted in Finland identified an inverse relationship between placental weight and the prevalence of type 2 diabetes in adulthood (p=0.002)[176]. Similarly, a higher placental weight/birth weight ratio was associated with a higher odds ratio of impaired glucose tolerance or type 2 diabetes in a study of 266 men and women aged 50 years [179]. These studies provide indirect evidence that placental size/transfer capacity plays an important role in the programming of later metabolic health.

There is also clear evidence from previous studies that maternal diet and nutritional status, especially in early gestation, can have significant effects on placental structure and function[45,46]. A prospective observational study of 538 mothers who delivered at term reported that high carbohydrate intake in early pregnancy and low dairy and meat protein intake later in pregnancy was associated with a lower placental weight at term[180]. In this study, placental weight decreased by 49g (95% confidence interval

16g to 81g, p=0.002) for each gram increase in the intake of carbohydrate in early pregnancy and by 1.4g (95% confidence interval 0.4g to 2.4g, p=0.005) for each gram decrease in protein intake in late pregnancy[180]. Another study which examined the association between under-nutrition during pregnancy and placental development in the Dutch Winter Hunger Famine cohort also reported that the combination of famine exposure in early pregnancy followed by higher food intake in mid-later pregnancy resulted in a higher placental weight at term than women from the same region who gave birth prior to the famine[181].

A number of animal studies have also provided evidence of substantial effects of maternal nutrition on placental growth, structure and function[182]. In a guinea pig model, Dwyer and colleagues reported that under-nutrition (60% *ad libitum*) in early pregnancy (until 15 days of gestation) resulted in a decreased interlobium weight (p=0.05) and increased placental weight/birth weight ratio (p=0.05) at both 45 days of gestation and at term[182]. Another study in guinea pigs also demonstrated that maternal under-nutrition during pregnancy could reduce placental weight and alter placental structure[183]. In this study, compared with guinea pigs fed *ad libitum*, animals fed 70% of *ad libitum* before and during pregnancy had decreased placental weight (p=0.001), total placental surface area (p=0.02 and 60 day of gestation, p<0.0005), the surface density of trophoblast (p=0.01) in mid and late pregnancy[183]. These changes in placental weight and structure would be expected to be associated with an impaired placental capability for substrate transfer, and were indicative of a delay in the structural maturation of the placenta[183,184]. Other experiments in sheep and rats have also suggested that maternal nutritional status during pregnancy is important for placental growth[185-187]. Kwong and colleagues reported that maternal

low protein (9% casein) diet during the preimplantation period (0-4.25 days after mating) decreased proliferation of the inner cell mass (both early and mid/late blastocyst) and trophectoderm lineages (mid/late blastocyst) in the placenta[187]. There is therefore clear evidence from both human studies and animal models that maternal nutritional status can impact on placental structure and function and that these changes may have an important role in mediating the effect of nutritional exposures *in utero* on offspring outcomes.

Despite significant evidence pointing to the ability of maternal n-3 LCPUFA supplementation to increase gestation length in humans, there are relatvively few mechanistic studies which have evaluated the role of n-3 LCPUFA/DHA in placental development and function. Recent studies have,however, provided evidence that n-3 LCPUFAs consumed in the diet are incorporated into placental cells, and can influence the balance of lipid-derived mediators in this organ. A rat study showed that maternal DHA supplementation from day 1 of pregnancy resulted in increased concentrations of DHA derived anti-inflammatory resolvins and protectins in the placenta on days 17 and 22 of gestation[188]. Similarly, in a randomised, placebo controlled trial, supplementation of n-3 LCPUFA (3.7g of n-3 LCPUFA containing 56.0% DHA and 27.7% EPA) from 20 week of gestation until birth increased placental levels of DHA by about 80% ($p<0.01$) and elevated placental concentrations of a number of physiologically significant anti-inflammatory lipid-derived mediators including 17-HDHA by about twofold ($p<0.005$) and 18-HEPE by threefold ($p<0.0001$), compared to women who took olive oil (placebo)[189]. DHA has also been reported to promote angiogenesis *in vitro*, which could enhance placental function by improving placental blood flow[190].

In addition, there is evidence that DHA can assist in resolving oxidative stress in the placenta, which is important given the role of placental oxidative stress in the pathophysiology of several pregnancy complications, including intrauterine growth restriction[191-194]. In rats, maternal n-3 LCPUFA supplementation from of the start pregnancy resulted in significant increases in labyrinth zone weight and decreases in labyrinth zone F-2 isoprostanes (a marker of oxidative damage) on both 17 and 22 d of gestation. Importantly, this was associated with a reduction in placental oxidative stress and consequent increase in placental and fetal growth[195]. Hence, previous studies have shown that increased maternal intake of n-3 LCPUFA during pregnancy can alter the balance of lipid-derived mediators and oxidative factors in the placenta which in turn would be expected to have positive effects on placental function. However, there is still limited understanding of whether and to what extent effects of DHA on the placenta may be involved in mediating the effects of increasing n-3 LCPUFA intake during pregnancy on offspring outcomes. Hence, further studies are needed to explore in greater detail how increased prenatal n-3 LCPUFA exposure impacts on the placenta, and how these changes relate to effects on offspring outcomes.

1.7. Summary

Obesity and type 2 diabetes are currently major public health issues in many countries across the world and the number of individuals affected by these diseases continues to increase. To prevent obesity and type 2 diabetes in adulthood, it is important to identify interventions which can be introduced early in life, before these conditions develop. Both human and animal studies have shown that there is association between the intrauterine environment and an increased risk of obesity, cardiovascular disease and type 2 diabetes in child and adult life. This early programming of poor metabolic health is thought to occur as a result of the ability of the nutritional environment experienced *in utero* to permanently alter the expression of genes in the offspring which regulate key metabolic processes. The mechanisms through which prenatal nutrition induces these gene expression changes is not clearly understood, however there is growing evidence that epigenetic processes have a critical role in mechanistic pathway through which prenatal nutritional environment impacts on the neonate's susceptibility to obesity in later life.

A large number of studies in rodents have shown that altering maternal nutrition affects the DNA methylation status of specific genes, and that these epigenetic modifications persist after birth and cause stable changes in gene expression in genes related to lipid and carbohydrate metabolism and appetite. Thus far, however, virtually all studies of the role of epigenetics in the programming of obesity have been carried out in animals, and knowledge of the role of epigenetics in the response of the fetus to nutritional exposures *in utero* and in the prenatal programming of obesity in humans is limited. Thus, the central aim of this thesis was to determine the effect of a specific nutritional intervention (maternal n-3 LCPUFA supplementation)

on global and gene-specific DNA methylation profiles in children at birth and at 5 years of age, and whether these changes were associated with markers of metabolic health (BMI, percent body fat, insulin sensitivity) in these same children at 5 years of age.

As shown in previous studies, maternal nutritional environment during pregnancy can have effects on placental structure and function[182]. Moreover, these placental changes have been reported to relate to measures of metabolic health in the offspring later in life[45,168,178]. As the most critical single organ contributing to fetal development, it has been suggested that that effects of maternal nutritional factors on the long-term health outcomes, including metabolic health outcomes, could potentially be mediated by effects on the placenta. In addition, there is accumulating evidence that increasing maternal DHA status can significantly alter the fatty acid composition of the placenta, and result in changes in the balance of pro-inflammatory/anti-inflammatory lipid mediators in this tissue. Despite this, however, there is currently limited understanding of how DHA affects placental growth/development and its impact on placental gene expression.

1.7.1. Specific Aims

While studies in adults and *in vitro* had suggested that n-3 LCPUFA could improve metabolic health outcomes, whether increasing the DHA supply before birth had benefits for the metabolic health of the children was unclear. The aim of Chapter 2 of this thesis, therefore, was to determine the effect of maternal n-3 LCPUFA supplementation on insulin sensitivity in the children at 5 years of age (an early marker of their type 2 diabetes risk). This aim was achieved as part of a 3 and 5 year follow-up of children whose mothers had participated in the DOMInO RCT, in which women were provided with either a high-dose n-3 LCPUFA supplement, chiefly as DHA, or placebo from 20 weeks gestation until delivery.

The aim of Chapter 3 and 4 of this thesis is to determine the impact of maternal DHA supplementation in pregnancy on global DNA methylation (Chapter 3) and gene-specific DNA methylation (Chapter 4) in the infants at birth and at 5 years of age. Global DNA methylation level (Chapter 3) and genome-wide DNA methylation level (Chapter 4) were assessed from blood samples collected in children at birth and at 5 years of age to determine the impact of maternal DHA supplementation during the second half of pregnancy on global DNA methylation and gene-specific genome-wide DNA methylation in children at birth and at 5 years. In Chapter 3, relationships between global DNA methylation status of the children at either birth and 5 years of age and measures of BMI, body fat mass or insulin sensitivity in early childhood were also investigated.

Other than epigenetic modifications, the placenta has been shown to be an important mediator of the effects of prenatal nutritional exposures on subsequent development.

Previous studies have shown that n-3 LCPUFA supplementation during pregnancy can affect size and structure, lipid-derived mediators and oxidative stress in the placenta which in turn would be expected to affect placental function. These alterations in placental structure and function can, in turn, alter pregnancy outcomes. However, there was limited understanding of the effect of DHA on placental development and function, and whether this could account for the impact of maternal n-3 LCPUFA supplementation on pregnancy and/or infant/child outcomes. Therefore, Chapter 5 of this thesis aimed to determine the impact of treatment with a DHA enriched fish oil emulsion on fatty acid composition, proliferation rate and gene expression profile in a human placenta first trimester cell line and to compare these effects with those resulting from treatment with a soy oil emulsion containing no DHA or other n-3 LCPUFA.

This thesis describes the results of studies which aimed to investigate the effects of specific nutritional exposure (n-3 LCPUFA) during pregnancy on epigenome of the children both at birth and in early childhood. Moreover, these studies attempt to relate these epigenetic changes to phenotypic effects. The results from this thesis provide new insights into the effects of prenatal n-3 LCPUFA supplementation on the infant/child and the mechanisms which could potentially underlie these effects.

CHAPTER 2: THE EFFECTS OF MATERNAL N-3 LCPUFA SUPPLEMENTATION ON INSULIN SENSITIVITY IN CHILDREN

2.1. Candidate Contribution

I was responsible for tracking all the samples collected as part of this clinical trial and undertook all sample processing and cataloguing. I validated the glucose and insulin assays to confirm their suitability for analysis of paediatric blood samples and undertook the glucose, insulin and fatty acid analyses for all samples in this clinical trial. I collated the results of these assays and calculated the HOMA-IR.

2.2. Introduction

The incidence of type 2 diabetes in Australia and worldwide has increased dramatically over the last decade, and continues to increase[1,2]. According to estimates made by the WHO, the number of type 2 diabetic individuals world-wide will reach 300 million by 2025 and this disease will be 7th leading cause of death by 2030[65,93]. This type 2 diabetes epidemic threatens to reduce the length and quality of life of current and future generations[65]. Hence, there is a need for safe and effective strategies for prevention which can feasibly be implemented on a population level[93]. As discussed in the literature review, there is evidence from both human and animal studies that the pathway to developing insulin resistance and type 2 diabetes begins very early in life, and that the nutritional environment an individual experiences before birth and in early infancy is a key determinant of their risk of later development of insulin resistance and type 2 diabetes[10,93]. This has led to the suggestion that nutritional interventions that are applied during the perinatal period are likely to be most effective in improving the long-term metabolic health of an individual, including their risk of type 2 diabetes[3-5,40-48]. To date, however, there have been few studies which have determined the effect of nutritional interventions applied during pregnancy and/or infancy on insulin sensitivity of individuals later in childhood.

In this context, there has been growing interest in the potential role of maternal n-3 LCPUFA supplementation to improve insulin sensitivity, and thus reduce the risk of type 2 diabetes, in the children. This is based on studies *in vitro* and in adult rodents which have suggested that the n-3 LCPUFA, in particular DHA and EPA, can reduce inflammation in adipose tissue and stimulate the synthesis and secretion of the

insulin sensitising hormone, adiponectin, from adipose cells and thereby improve peripheral insulin sensitivity[196]. While the data from human studies has been less consistent, some studies have also reported beneficial effects of n-3 LCPUFA supplementation in adults on several features of the metabolic syndrome including insulin resistance. In one study, Mori and colleagues found that a supplement providing 3.65g/day n-3 LCPUFA in conjunction with an energy restricted diet for 16 weeks was associated with improvements in insulin sensitivity in overweight and obese adults that were significantly greater than those given the energy restricted diet alone[197]. In another study, consuming supplements containing 3g DHA + EPA per day for 8 weeks significantly increased insulin sensitivity in type 2 diabetic subjects[198]. Similarly, Tsitouras and colleagues found that 8 weeks of dietary intake of 720g/week of fatty fish and 15mL/day sardine oil significantly increased insulin sensitivity in men and women over 60 years of age (n=12)[199]. Despite these encouraging results, however, no studies have determined whether increasing the supply of n-3 LCPUFA during the critical period of fetal/infant development can improve subsequent insulin sensitivity, and thereby reduce the susceptibility to type 2 diabetes in child and adulthood.

Therefore, the aim of this Chapter was to determine the effect of maternal supplementation with n-3 LCPUFAs, mainly as DHA, during the second half of pregnancy on insulin sensitivity of their children at 5 years of age. This study was performed as part of a follow-up of a large double blinded randomised controlled trial, the DOMInO trial[128].

2.3. Materials and Methods

2.3.1. Study Population

The present project was nested within a follow-up study of a subset of children whose mothers participated in the DOMInO trial[128].

2.3.2. The DOMInO Trial

DOMInO is the largest double-blinded randomised placebo-controlled trial to date to investigate the impact of maternal supplementation with n-3 LCPUFA, predominately as DHA, in the second half of pregnancy. The DOMInO trial was registered at www.anzctr.org.au as ACTRN12605000569606. A total of 2,399 women were enrolled from 5 centres across Australia, of which 1,660 were enrolled at Adelaide-based centres including the Women's and Children's Hospital (WCH), Flinders Medical Centre (FMC) and private hospitals. Women were eligible to participate if they had a singleton pregnancy and were less than 20 weeks of gestation. Women who were already taking supplements containing DHA, had a known fetal abnormality, had a bleeding disorder in which tuna oil was contraindicated, were taking anticoagulant therapy, had a history of drug or alcohol abuse or for whom English was not the main language spoken in the home were excluded[128].

All women recruited into the DOMInO study were randomly assigned to either the treatment group (DHA) (n=1,197) or control (placebo) (n=1,202) group. Women in the treatment group were asked to consume 3×500mg capsules which contained DHA-rich fish oil daily, and which provided 800mg DHA and 100mg EPA per day. The women in the control group consumed 3×500mg capsules of vegetable oil without DHA (placebo). The composition of the placebo capsules was designed to match with the average dietary fatty acids profile in the typical Australian diet. All women took their assigned capsules from ~20 weeks gestation until the birth of their child, and compliance was 97.3% in the DHA group and 96.1% in the control group[128]. There were no differences between the treatment groups in demographic

and clinical characteristics at baseline indicating that the randomisation process was appropriate[128].

2.3.3. Growth and Insulin Resistance Follow Up of the DOMInO Children

The growth and insulin resistance follow-up at 3 and 5 years included the children of participants who were recruited at any of the DOMInO's Adelaide centres (WCH, FMC and private hospitals). The study was approved by Children, Youth and Women's Health Service Research Ethics Committee and Flinders Medical Centre Research Ethics Committee. All eligible participants (n=1,618 of the original sample of 1,660 DOMInO participants in Adelaide) were sent an information sheet and consent form when the children were two and half years of age. The follow-up study commenced in March 2009 and was completed in October 2013. 1,516 participants consented to the study, and 1,414 children (93.2% of consenters) completed the clinic appointment at 5 years of age. All staff and researchers involved in the 3 and 5 year DOMInO follow up study were blinded to group allocation until the end of the study.

2.3.4. 5 Year Clinic Assessments

Anthropometric measurements including weight, height, blood pressure and head-, waist- and hip-circumferences of the children were collected by trained research staff at the 5 year appointment and recorded in each participant's Case Report Form. Total fat mass and percentage body fat was measured using Bioelectrical Impendence Spectroscopy (BIS). A 5mL blood sample was taken from 714 children at 5 years of age into a 10mL purple cap EDTA vacuum tube. After collection, the blood samples were stored at 4ºC before being transferred to the laboratory for processing. Each sample was labelled with study ID, subject's name and sex, date of birth, date and time of collection. Every sample was recorded in a bar-code tracking system to trace the location, analysis details and other information. The sample collection commenced in May 2011 and was completed in July 2013.

2.3.5. Processing and Storage of Blood Samples

Whole blood samples were centrifuged at 3,200rpm for 10 mins to separate red blood cells (RBC), plasma and buffy coat within 24 hours of collection. The clear upper plasma layer was placed into 4 separate cryotubes and stored at -80ºC for subsequent analysis of glucose and insulin concentrations. The buffy coat was placed in a separate cryotube and stored at -80ºC for subsequent DNA extraction. The lower RBC layer was mixed with 0.9% saline and stored at 4ºC for processing for fatty acid analysis within 48 hours. I undertook the blood sample processing for all the samples collected in the follow-up study. The number of samples on which glucose, fatty acid composition and insulin analysis were conducted is shown in Figure 2.1.

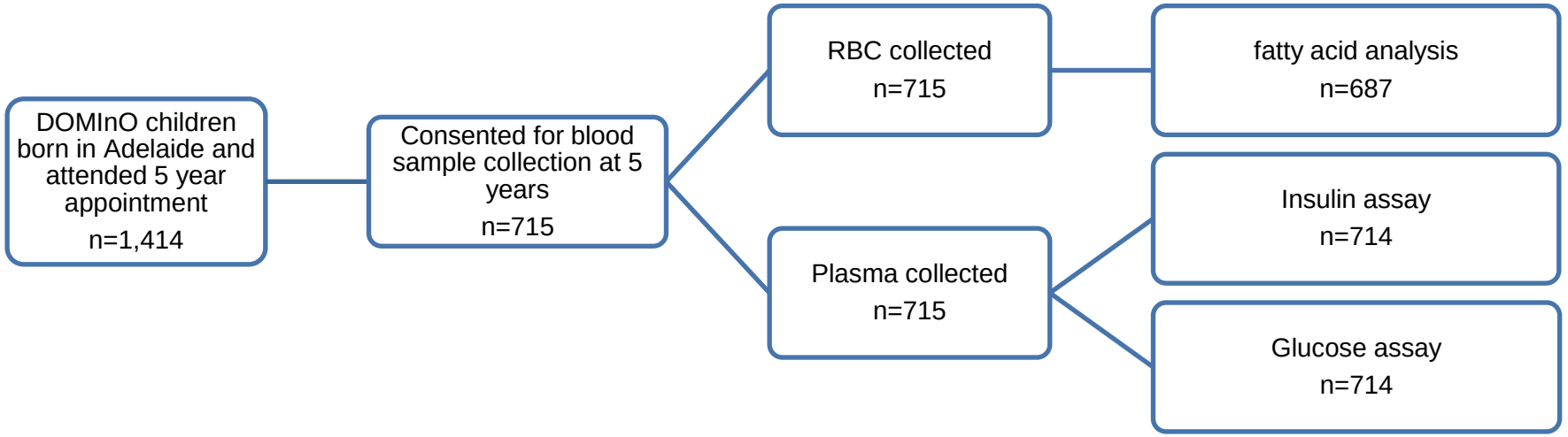

Figure 2.1: Diagram showing the number of samples used for fatty acid analysis, and insulin and glucose measurements from the blood samples collected from DOMInO children at 5 years of age.

2.3.6. Fatty Acid Analysis

2.3.6.1.Fatty Acid Extraction from RBC

Of the 715 blood samples collected at 5 years, 687 had a sufficient volume of red blood cells remaining after processing for the analysis of fatty acid composition. Of these samples, 6 produced physiologically implausible results, and were excluded from the analysis. Total lipids were extracted from RBC with chloroform/isopropanol (2:1, v/v). Butylated hydroxyanisole (BHT, 0.005%, w/v) was added to all organic solvents except n-heptane to prevent fatty acid oxidation.

Within 24 hours of sample collection, RBC were washed 3 times with cold 0.9% saline to remove any residual plasma or buffy coat. 0.5mL of the RBC was then transferred into a 10mL plastic centrifuge tube, followed by the addition of 1mL 0.9% saline and 2mL isopropanol. The samples were mixed by inversion, and then 4mL chloroform was added to each sample. The samples were mixed vigorously and then allowed to stand at room temperature for at least 5 mins before being centrifuged for 10 mins at 3,000rpm to separate the aqueous and organic solvent phases. The bottom organic layer was then transferred carefully into a labelled 20mL glass scintillation vial using a disposable glass pasteur pipette and evaporated to dryness using a nitrogen evaporator. 150µL of chloroform/methanol (9:1) was then added to the vial for further total lipid separation.

2.3.6.2.Lipid Classes Separation by Thin Layer Chromatography

The phospholipid and triglyceride fractions were separated from total lipid extracts by thin layer chromatography (TLC) on silica gel plates (Silica gel 60H, Merck,

Darmstadt, Germany). A TLC standard 18-5 (Nu-Chek Prep Inc, MN, USA) was run on the plates for lipid identification. The mobile phase for TLC was petroleum spirit/acetone (3:1, v/v). The TLC plates were sprayed with fluorescein 5-isothiocyanate in methanol, and the lipid classes present were visualised under UV light. The phospholipid fraction remained at the bottom of TLC plates while other fatty acids fractions migrated to the upper portion of the plate. The phospholipid fractions for each sample were scraped and transferred individually into vials containing 2mL 1% (v/v) H_2SO_4 (18M AR grade, BDH, Sussex, UK) in anhydrous methanol in a 5mL sealed vial (Wheaton, Millville, USA) for transmethylation at 70ºC for 3 hours. After cooking and cooling the vial to room temperature, 0.25mL of distilled water and 0.75mL of heptane was added into the bottle, and mixed by vortexing. The upper clear heptane layer, containing fatty acid methyl esters (FAME), was transferred into a 2mL Agilent Gas Chromatograph (GC) auto-injector vial containing a few grams of anhydrous Na_2SO_4 (dehydrating agent) and sealed for analysis by gas chromatography.

2.3.6.3.GC Analysis of Fatty Acid Methyl Esters

FAME were separated and quantified using a GC (Hewlett-Packard 6890; Palo Alto, CA, USA) equipped with a BPX70 capillary column 50m×0.32mm, film thickness 0.25µm (SGC Pty Ltd., Victoria, Australia), programmed temperature vaporisation injector (PTV) and a flame ionisation detector (FID). The PTV temperature was set at 250ºC and FID temperature at 300ºC, a programmed temperature ramp (140-240ºC) was used. Helium gas was used as a carrier at a flow rate of 35cm per second in the column and the inlet split ratio was set at 20:1. The identification and

quantification of FAME was achieved by comparing the retention times and peak area values of unknown samples to those of commercial lipid standards (Nu-Chek Prep Inc., Elysian, MN, USA) using the Hewlett-Packard Chemstation data system. This method is routinely used for the measurement of fatty acid composition in human plasma samples in the FOODplus group[128].

2.3.7. Infinity Glucose Hexokinase Assay

Of the 715 samples collected from the DOMInO children at 5 years of age, 714 underwent analysis for glucose and insulin concentrations. The remaining sample was of insufficient volume (total blood volume less than 0.1mL) to perform these assays. Plasma concentrations of glucose were determined using the Infinity Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA, USA) following the manufacturer's instructions. Assays and analyses were conducted using a Konelab 20 (Thermo Scientific, Vantaa, Finland). Quality controls were tested with every batch of samples. The inter- and intra-assay coefficients of variation were both <10%. Each sample was tested in duplicate.

2.3.7.1.Validation of Infinity Glucose Hexokinase Kit

Methods of measuring glucose level in plasma were validated in our laboratory in accordance with the European Medicine Agency ICH topic Q2 (R1) Validation of Analytical Procedures [200] based on 5 tests: linearity, specificity, parallelism, precision and detection and reporting limits.

2.3.7.1.1. Linearity

For the linearity assessment, 8.5 and 6.5mmol/L stock glucose solutions provided by the kit manufacturer (Thermo Electron, Pittsburgh, PA, USA) were diluted by factors of 1, 1.25, 1.67, and 2.5 to generate a dilution series. These diluted concentrations were expected to cover the range of plasma glucose levels that would be present in the majority of this study population[201]. These diluted samples were then all run in a single assay and a graph of dilution factor (x axis) vs assay result (y axis) plotted for each sample using Microsoft Excel. The equation and R^2 value for the line of best fit

for each of these graphs was determined using the software built into this program. Slope, y-intercept, slope uncertainty and intercept uncertainty, residue and R^2 were used to evaluate the degree of linearity.

2.3.7.1.2. Specificity

In order to test the specificity of the assay, a recovery test was conducted in which samples of unknown glucose concentration were spiked by the addition of a known amount of glucose. A 600µL volume of two plasma samples with unknown glucose concentrations were spiked with 100µL of a glucose solution with a concentration of 4.5mmol/L. This spiked solution was then serially diluted by combining equal volumes of the previous dilution and un-spiked plasma to make up two additional solutions. The glucose levels in the un-spiked solution, spiked solution and three diluted solutions were then measured in the glucose assay.

The expected glucose level in the first spiked solution was calculated (expected concentration) according to the equation:

$$Expected\ concentration\ in\ spiked\ solution = \frac{amount\ of\ glucose\ in\ unspiked\ solution + amount\ of\ glucose\ added\ to\ sample}{total\ volume}$$

All subsequent serial dilutions were at a ratio of 1:1 of the preceding dilution and the un-spiked plasma sample. Therefore, for the first dilution, the concentration could be calculated as the average of the expected concentration of spiked and concentration of un-spiked solution.

The same principle was applied for all subsequent dilutions.

$$Concentration\ of\ dilution = average\ [C\ (unspiked) and\ C\ (spiked)]$$

Once the expected concentrations of spiked and diluted solutions were calculated, the efficiency of the assay was calculated by determining the percentage recovery according to the equation:

$$Recovery = \frac{Observed\ concentration}{Expected\ concentration}$$

Where the observed value is the measurement obtained in the assay and the expected concentration is the calculated value based on the amount of glucose added.

2.3.7.1.3. Accuracy

The method was validated for accuracy by comparing the glucose level obtained for five replicates of three glucose solutions at concentrations of 6, 4.5 and 3mmol/L provided by the manufacturer (Thermo Electron, Pittsburgh, PA, USA) in one assay. The relative standard deviation (RSD) of repeatability, 95% confidence interval (CI) and recovery rate (measured value/provided concentration from the manufacturer) were calculated to assess the accuracy of the assay.

2.3.7.1.4. Precision

The precision test consisted of two parts: intra- and inter-assay. In order to determine the intra-assay coefficient of variation (CV), three samples with different glucose level were run in four replications in a single assay. Two samples were run in triplicate in three separate assays all conducted on different days in order to determine the inter-assay CV.

2.3.7.1.5. Limit of Detection (LOD), Method Quantitation Limit (MQL) and Instrument Quantitation Limit (IQL)

LOD refers to the lowest concentration where it is possible to distinguish a signal from the background. For this study, the definition of the limit of detection was set by the International Union of Pure and Applied Chemistry[202] and the National Association of Testing Authorities, Australia[203]. According to this definition, LOD was calculated as the mean concentration plus 3 times the standard deviation of the concentration of a calibration blank measured in the same assay at least seven times. In our study, 10 calibration blanks were measured to determine LOD.

The MQL is defined as the minimum concentration of an analyte that can be measured within specific limits of precision and accuracy and is calculated as 3×LOD multiplied by the dilution factor. IQL is calculated as 3×LOD.

2.3.8. Insulin Assay

Plasma insulin levels in the samples collected from the DOMInO children were tested using the ALPCO Immunoassay Ultrasensitive Insulin ELISA kits (ALPCO Diagnostics, NH, USA) following the manufacturer's instructions. Briefly, 25µL plasma and 100µL Detection Antibody were added into each well of a 96-well microplate followed by a 1 hour incubation at room temperature with shaking at 700-900rpm on a Platform Mixer (AdeLab, Adelaide, Australia). The microplate wells were washed 6 times with 350µL of working strength wash buffer (ALPCO Diagnostics, NH, USA). 100µL of TMB Substrate was then added into each well followed by a 30-min incubation at room temperature on a Platform Mixer at 700-900rpm. After 30 mins, 100µL of Stop Solution was added into each well to stop the reaction, and the microplate was read by a calibrated BioTek EL808 microplate reader (BioTek Instruments, USA) at a wavelength of 450nm. In every analytical run, the same standards (0, 0.15, 1, 3, 10, 20µIU/mL), 1 reconstituted control and 2 plasma samples (as quality controls) were tested on each plate. Each sample was tested in triplicate.

2.3.8.1.Validation of ALPCO Immunoassay Ultrasensitive Insulin ELISA Assay

The ALPCO Immunoassay Ultrasensitive Insulin ELISA kit was validated in our lab in accordance with the European Medicine Agency ICH topic Q2 (R1) Validation of Analytical Procedures [200] using same approach as for the Infinity Glucose Hexokinase kit. As mentioned previously, five tests were included in the validation: linearity, specificity, parallelism, precision and detection and reporting limit.

2.3.8.1.1. Linearity

For the linearity assessment, two stock solutions with unknown concentrations (ALPCO Diagnostics, NH, USA) were diluted by factors of 1, 1.25, 1.67, and 2.5 to generate a dilution series. The slope, y-intercept, and R^2 of the line of best fit of the plot of measured insulin concentration and dilution factor were determined to evaluate linearity.

2.3.8.1.2. Specificity

In order to test the specificity of the assay, two plasma samples (48µL) of unknown insulin concentration from volunteers were spiked with standard insulin solution (12µL) which contained 20µIU/mL insulin (provided by the manufacturer, ALPCO Diagnostics, NH, USA). This spiked solution was then serially diluted to generate two further solutions. The specificity was determined following the same principle as described above for the glucose assay.

2.3.8.1.3. Accuracy

The method was validated for accuracy by comparing the insulin level obtained for five replicates of one commercial insulin solution with concentrations ranging from 7.98µIU/mL to 10.39µIU/mL (ALPCO Diagnostics, NH, USA) in one assay. The RSD of repeatability and 95% confidence interval (CI) were calculated to check the accuracy.

2.3.8.1.4. Precision

The precision test consisted of two parts: intra- and inter-assay. In order to determine the intra-assay CV, 6 samples with different insulin concentrations were run in

triplicate in a single assay. Five samples were run in triplicate in seven separate assays all conducted on different days in order to determine the inter-assay CV.

2.3.8.1.5. LOD, MQL and IQL

Ten calibration blanks were measured to determine limit of detection. The same equation and calculation methods as in the glucose validation were used to determine the LOD, MQL and IQL for the insulin assay.

2.3.9. Assessment of Insulin Sensitivity, the HOMA-IR

The HOMA-IR was used to provide a measurement of insulin resistance in all DOMInO children. HOMA-IR was calculated from the fasting glucose and insulin concentrations according to the equation:

$$HOMA - IR = \frac{[fasting\ glucose\ (mmol/L)] \times [insulin\ (\mu IU/mL)]}{22.5}$$

2.3.10. Statistical Analysis

The statistical analyses for the DOMInO 3 and 5 year growth and insulin resistance follow-up study were conducted by the Data Management and Analysis Centre (DMAC) at the University of Adelaide, and were conducted using SAS version 9.3 (Cary, NC, USA). All analyses were performed on an intention-to-treat basis. Multiple imputation was performed separately by treatment group using chained equations to create 100 complete datasets for analysis, under the assumption that data were missing at random. The primary analysis was based on imputed data and included all participants who consented to the follow-up study. Sensitivity analyses were performed on the available data and on imputed data for all 1,660 children born to women enrolled in Adelaide-based centres. All analyses produced similar results and only the results of the primary (imputed) analysis are included in this Chapter.

The effect of maternal DHA supplementation on continuous outcomes, including anthropometric measures, plasma glucose and insulin and HOMA-IR were analysed using linear regression models, with treatment effects expressed as differences in

means. The HOMA-IR values in the dataset were skewed and were therefore log transformed prior to analysis. A priori secondary analyses were performed to test for effect modification by sex.

All analyses were adjusted for the stratification variables for the DOMInO trial, centre and parity, as well as child sex and PPARγ genotype and mother's secondary education, further education, smoking status and BMI at baseline. The PPARγ single-nucleotide polymorphism is strongly related to risk of obesity/type 2 diabetes and has a population prevalence of approximately 20%[204]. Statistical significance was assessed at the 2-sided $p<0.05$ level.

2.4. Results

2.4.1. Validation of Infinity Glucose Hexokinase Assay

There was a linear relationship between glucose concentration and dilution factor with the R^2 for the line of best fit for both samples being close to 1 (Figure 2.2). The recovery rate of observed/expected glucose concentrations were between 95.7% and 98.7%, indicating a high degree of specificity (Table 2.1). Glucose solutions at concentrations of 6, 4.5 and 3mmol/L produced average recovery rates of 101.01%, 101.19% and 100.13% respectively when they were tested five times (Table 2.2). The high recovery rates indicated that the glucose assay has the ability to provide an accurate indication of glucose concentration in human plasma samples. The intra- (Table 2.3) and inter-assay (Table 2.4) CV were 3.2% and 5.7%, respectively, which indicated a high degree of precision and little variation in results obtained for the same sample when it was run either multiple times in the same assay or in different assays on separate days. The LOD of the assay, calculated from the results of ten blanks (MilliQ water) was 0.026mmol/L. Since the dilution factor of measured solution was 1, the IQL was equal to the MQL, which was 0.078mmol/L.

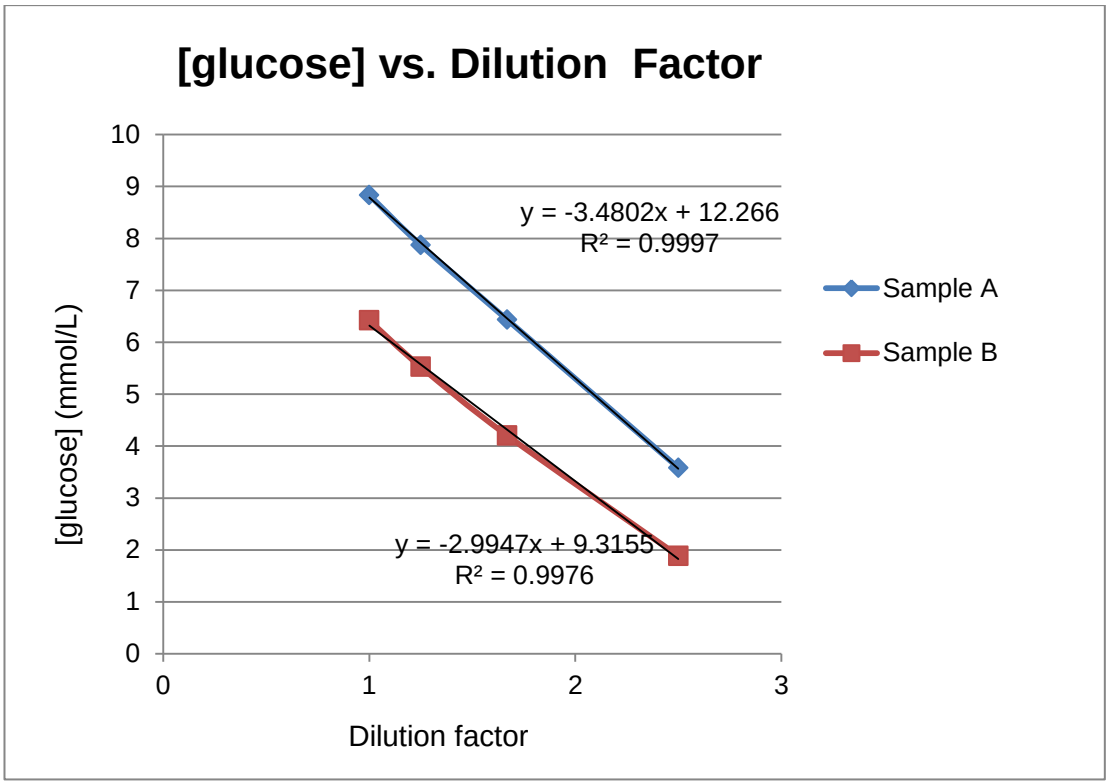

Figure 2.2: Scatter plot of glucose concentration measured in the glucose assay (y axis) against sample dilution factor (x axis) for 2 plasma samples. The linear trend lines show a significant negative relationship between glucose concentrations and dilution factor, with R^2 of 0.9997 and 0.9976 respectively.

Table 2.1: Specificity test results for the Infinity Glucose Hexokinase assay validation

Sample		*Observed [glucose]*	*Expected [glucose]*	*Recovery (%)*
Sample A	Un-spiked sample	4.773	-	-
	Spiked sample	4.522	4.682	96.6
	Dilution 1	4.610	4.727	97.5
	Dilution 2	4.637	4.750	97.6
Sample B	Un-spiked sample	5.330	-	-
	Spiked sample	4.892	5.053	96.8
	Dilution 1	4.969	5.191	95.7
	Dilution 2	5.197	5.260	98.8

[glucose]: glucose concentration (mmol/L).

Table 2.2: Accuracy results for Infinity Glucose Hexokinase assay

Sample	*[glucose] 1*	*[glucose] 2*	*[glucose] 3*	*[glucose] 4*	*[glucose] 5*	*Mean*	*CV%*	*Expected [glucose]*	*Recovery (%)*
A	6.087	6.091	6.114	6.121	5.891	6.061	1.6%	6	101.01
B	4.668	4.529	4.497	4.567	4.507	4.554	1.5%	4.5	101.19
C	3.002	2.879	3.113	3.015	3.011	3.004	2.8%	3	100.13

[glucose]: glucose concentration (mmol/L).
CV: coefficient of variation

Table 2.3: Intra-assay CV of three samples in same batch

Sample	*[glucose]1*	*[glucose]2*	*[glucose]3*	*[glucose]4*	*Mean*	*SD*	*CV%*
A	8.646	8.307	8.583	8.537	8.518	0.148	1.7%
B	6.881	6.807	6.267	6.798	6.688	0.283	4.2%
C	4.529	4.222	4.179	4.222	4.288	0.162	3.8%
Average CV%							3.2%

[glucose]: plasma glucose concentration (mmol/L).
SD: standard deviation.
CV: coefficient of variation.

Table 2.4: Inter-assay CV of two samples in three separate assays

Sample	*[glucose] 1*	*[glucose] 2*	*[glucose] 3*	*Mean*	*SD*	*CV%*
A	5.118	5.015	5.570	5.234	0.295	5.6%
B	5.315	5.585	4.971	5.290	0.308	5.8%
Average CV%:						5.7%

[glucose]: plasma glucose concentration (mmol/L)
SD: standard deviation.
CV: coefficient of variation.

2.4.2. Validation of ALPCO Immunoassay Ultrasensitive Insulin ELISA Assay

There was a negative curvilinear relationship between insulin concentration and dilution factor which was parallel to the assay standard curve with the R^2 of the linear trend lines of both samples close to 1 (Figure 2.3). The recovery rates as determined by observed/expected insulin concentrations in the recovery test were between 92% and 107%, indicating a high degree of specificity (Table 2.5). The insulin concentrations obtained for the commercial insulin solution were within the acceptable range provided by the manufacturer (Table 2.6). The high recovery rate indicated that the ALPCO Immunoassay Ultrasensitive Insulin ELISA assay had the ability to provide an accurate measure of insulin concentrations in human plasma samples. The intra- (Table 2.7) and inter-assay (Table 2.8) CV were 1.9% and 13.8%, respectively. Ten blanks were tested to obtain the LOD for the assay, which was 0.028mmol/L; and, the IQL which was equal to MQL was 0.084 mmol/L.

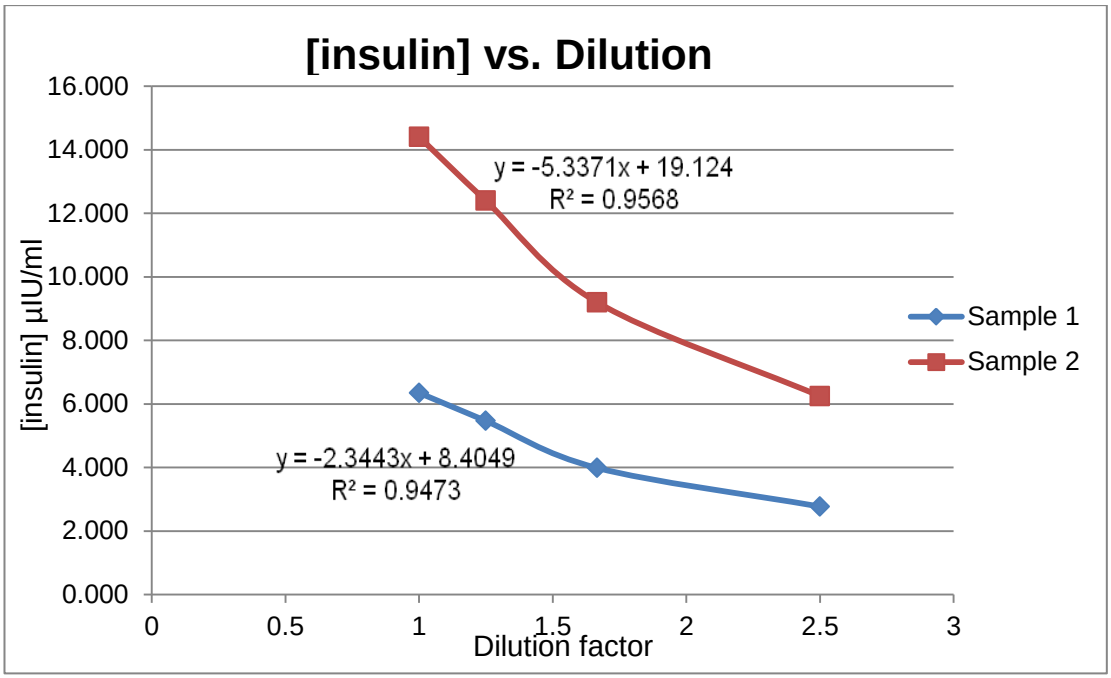

Figure 2.3: Scatter plot of insulin concentration measured in the insulin assay (y axis) against sample dilution factor (x axis) for 2 plasma samples. The curve trend lines indicate significant negative relationships between insulin concentrations and dilution factor, with R^2 of 0.9473 and 0.9586 respectively.

Table 2.5: Specificity test results for the ALPCO Immunoassay Ultrasensitive Insulin ELISA assay validation

Sample		***Observed [insulin]***	***Expected [insulin]***	***Recovery (%)***
Sample A	Un-spiked sample		-	-
	Spiked sample	7.7	8.0	96
	Dilution 1	6.8	7.4	92
	Dilution 2	6.7	7.2	95
Sample B	Un-spiked sample		-	-
	Spiked sample	15.0	14.6	103
	Dilution 1	14.9	14.3	105
	Dilution 2	15.2	14.9	107

[insulin]: insulin concentration (µIU/mL).

Table 2.6: Accuracy test results for ALPCO Immunoassay Ultrasensitive Insulin ELISA assay.

	[insulin] 1	*[insulin] 2*	*[insulin] 3*	*[insulin] 4*	*[insulin] 5*	*Mean*	*CV%*
Sample	8.171	8.786	8.749	9.576	8.665	8.390	9.5%

[insulin]: insulin concentration (µIU/mL).
CV: coefficient of variation.
The accepted range specified for this external standard by the manufacturer was between 7.969-10.386.

Table 2.7: Intra-assay CV of six samples in same batch.

Sample	*[insulin] 1*	*[insulin] 2*	*[insulin] 3*	*Mean*	*SD*	*CV (%)*
A	0.136	0.130	0.132	0.133	0.003	2.3%
B	0.152	0.152	0.153	0.152	0.001	4.0%
C	0.304	0.304	0.306	0.305	0.001	4.0%
D	0.596	0.550	0.578	0.575	0.023	4.0%
E	1.598	1.599	1.601	1.599	0.002	0.1%
F	3.074	2.882	2.875	2.944	0.113	3.8%
Average CV						1.9%

[insulin]: plasma insulin concentration (µIU/mL).
SD: Standard deviation.
CV: coefficient of variation.

Table 2.8: Inter-assay CV of five samples in seven different days.

Sample	*[insulin] Batch 1*	*[insulin] Batch 2*	*[insulin] Batch 3*	*[insulin] Batch 4*	*[insulin] Batch 5*	*[insulin] Batch 6*	*[insulin] Batch 7*	*[insulin] Mean*	*SD*	*CV (%)*
1	0.020	0.032	0.016	0.022	0.022	0.020	0.022	0.022	0.005	22.7%
2	0.172	0.156	0.124	0.134	0.144	0.113	0.142	0.141	0.020	14.1%
3	0.437	0.421	0.336	0.387	0.372	0.321	0.352	0.375	0.043	11.5%
4	1.451	1.345	1.064	1.121	1.125	1.209	1.272	1.226	0.138	11.3%
5	2.812	2.674	2.329	2.159	2.331	2.619	2.409	2.476	0.231	9.3%
Average CV										13.8%

[insulin]: plasma insulin concentration (µIU/mL).
S.D: Standard deviation.
CV: co-efficient of variation.

2.4.3. Characteristics of the DOMInO Participants at Enrolment

As published previously, there were no differences in maternal baseline characteristics, including maternal weight, height, BMI and gestational age at enrolment into the DOMInO trial between women randomised to the DHA or control treatment groups[128] and there were also no differences in these parameters between groups in the sub-sample who participated in the 3 and 5 year growth and insulin resistance follow-up (Table 2.9). In addition, lifestyle factors, including maternal drinking, smoking and intake of non-DHA containing nutritional supplements, were not different between the two treatment groups both in the whole DOMInO population[128] and in the sub-sample selected from the follow-up study (Table 2.9). Socioeconomic factors, including maternal ethnicity, maternal education and maternal occupation and the number of male and female children were also not different between the treatment groups[128] (Table 2.9).

Table 2.9: Baseline characteristics in the sub-sample of DOMInO participants who participated in the 3 and 5 year growth and insulin resistance follow-up.

Characteristic	***DHA*** *(n=770)*	***Control*** *(n=761)*	***Total*** *(n=1531)*
Maternal Weight (kg): Median (IQ range)	72.0 (64.0-83.1)	72.0 (62.0-82.8)	72.0 (63.0-83.0)
Maternal Height (cm): Median (IQ range)	165.0 (160.0-169.0)	164.0 (160.0-169.0)	165.0 (160.0-169.0)
Maternal BMI at Baseline: Median (IQ range)	26.2 (23.5-30.1)	26.3 (23.2-30.5)	26.3 (23.4-30.4)
Maternal BMI Category at Baseline: N (%)			
. Missing	4 (0.5)	6 (0.8)	10 (0.7)
. Underweight	11 (1.4)	9 (1.2)	20 (1.3)
. Normal Weight	273 (35.5)	288 (37.8)	561 (36.6)
. Overweight	285 (37.0)	247 (32.5)	532 (34.7)
. Obese	197 (25.6)	211 (27.7)	408 (26.6)
Gestational Age at Randomisation: Median (IQ range)	19.0 (19.0-20.0)	19.0 (19.0-20.0)	19.0 (19.0-20.0)
Number of Previous Births: N (%)			
. 0	322 (41.8)	323 (42.4)	645 (42.1)
. 1-2	381 (49.5)	374 (49.1)	755 (49.3)
. 3-4	55 (7.1)	55 (7.2)	110 (7.2)
. 5 or more	12 (1.6)	9 (1.2)	21 (1.4)
Mother Was Current Smoker at Enrolment: N (%)			
. 1 - 20 per day	106 (13.8)	104 (13.7)	210 (13.7)
. 21 - 40 per day	4 (0.5)	4 (0.5)	8 (0.5)
. No	660 (85.7)	653 (85.8)	1313 (85.8)
Mother Smoked Leading Up to Pregnancy: N (%)			
. 1 - 20 per day	175 (22.7)	218 (28.6)	393 (25.7)
. 21 - 40 per day	37 (4.8)	28 (3.7)	65 (4.2)
. No	558 (72.5)	514 (67.5)	1072 (70.0)
. Missing	0 (0.0)	1 (0.1)	1 (0.1)
Mother Was Current Drinker: N (%)			
. Up to 1 to 2 per week	74 (9.6)	70 (9.2)	144 (9.4)
. Up to 7 - 10 per week	0 (0.0)	4 (0.5)	4 (0.3)
. No	696 (90.4)	687 (90.3)	1383 (90.3)
Mother Drank Leading Up to Pregnancy: N (%)			
. Up to 1 to 2 per week	305 (39.6)	333 (43.8)	638 (41.7)
. Up to 7 - 10 per week	155 (20.1)	140 (18.4)	295 (19.3)
. More than 10 per week	26 (3.4)	20 (2.6)	46 (3.0)
. No	284 (36.9)	268 (35.2)	552 (36.1)
Mother Further Education: N			

(%)			
. Certificate/Diploma	321 (41.7)	355 (46.6)	676 (44.2)
. Degree	160 (20.8)	147 (19.3)	307 (20.1)
. Higher Degree	34 (4.4)	31 (4.1)	65 (4.2)
. None	255 (33.1)	228 (30.0)	483 (31.5)
Father Further Education: N (%)			
. Certificate/Diploma	329 (42.7)	352 (46.3)	681 (44.5)
. Degree	120 (15.6)	118 (15.5)	238 (15.5)
. Higher Degree	35 (4.5)	31 (4.1)	66 (4.3)
. Missing	26 (3.4)	26 (3.4)	52 (3.4)
. None	260 (33.8)	234 (30.7)	494 (32.3)
Infant Gender: N (%)			
. Female	384 (49.9)	382 (50.2)	766 (50.0)
. Male	386 (50.1)	379 (49.8)	765 (50.0)

IQ: inter-quartile range

BMI: body mass index

2.4.4. Anthropometric Measurements of the DOMInO Children at 5 years of Age

There was no differences in body weight, body weight z-score, body fat percentage, BMI or BMI z-score at 5 years of age between treatment groups, either in the whole sample or when male and female children were analysed separately (Table 2.10).

Table 2.10: Anthropometric measurements of the DOMInO children at 5 years of age

Outcome	*DHA (n=770)*	*Control (n=761)*	*p value*
Body Weight at 5 Years (kg): mean (SD)			
. Male	20.19 (2.90)	20.12 (2.99)	0.85
. Female	19.71 (3.08)	19.61 (3.13)	0.81
. Total	19.95 (3.00)	19.87 (3.07)	0.68
Weight z-score at 5 Years: mean (SD)			
. Male	0.55 (0.99)	0.51 (0.97)	0.58
. Female	0.36 (0.96)	0.33 (0.96)	0.57
. Total	0.45 (0.98)	0.42 (0.97)	0.43
Body Fat Percentage at 5 Years: mean (SD)			
. Male	20.52 (6.23)	20.99 (6.09)	0.38
. Female	26.41 (6.08)	25.82 (6.17)	0.21
. Total	23.46 (6.82)	23.42 (6.59)	0.75
BMI at 5 Years (kg/m^2): mean (SD)			
. Male	16.20 (1.54)	16.19 (1.73)	0.96
. Female	16.17 (1.68)	16.20 (1.73)	0.90
. Total	16.19 (1.61)	16.20 (1.73)	0.99
BMI z-score at 5 Years: mean (SD)			
. Male	0.63 (0.98)	0.59 (1.07)	0.60
. Female	0.49 (0.95)	0.50 (0.98)	0.95
. Total	0.56 (0.97)	0.54 (1.03)	0.66

SD: standard deviation

BMI: body mass index

2.4.5. Fatty Acids Status of the DOMInO Children at 5 Years of Age

There was no significant difference in linoleic acid (LA), arachidonic acid (AA), EPA, docosapentaenoic Acid (DPA) and DHA status at 5 years of age between the treatment groups in either males or females, or in the population overall (Table 2.11).

Table 2.11: Fatty acids status of the DOMInO children at 5 years of age.

	DHA (n=335)	***Control (n=346)***	***P value***
LA (%): mean (SD)			
. Total	10.78 (1.30)	10.93 (1.25)	0.15
. Male	10.74 (1.26)	10.99 (1.19)	0.17
. Female	10.81 (1.34)	10.87 (1.31)	0.55
AA (%): mean (SD)			
. Total	13.00 (1.36)	13.06 (1.31)	0.24
. Male	12.98 (1.35)	12.96 (1.28)	0.84
. Female	13.03 (1.36)	13.15 (1.32)	0.24
EPA (%):mean (IQ range)			
. Total	0.63 (0.18)	0.62 (0.17)	0.30
. Male	0.66 (0.17)	0.65 (0.18)	0.46
. Female	0.65 (0.18)	0.63 (0.17)	0.14
DPA (%): mean (SD)			
. Total	2.51 (0.39)	2.47 (0.35)	0.62
. Male	2.55 (0.39)	2.51 (0.34)	0.15
. Female	2.46 (0.38)	2.44 (0.35)	0.48
DHA (%):mean (IQ range)			
. Total	3.57 (0.92)	3.57 (0.83)	0.62
. Male	3.73 (0.92)	3.78 (0.87)	0.47
. Female	3.71 (0.89)	3.58 (0.83)	0.07

%: percentage of total fatty acids

SD: standard deviation

IQ: inter-quartile range

2.4.6. Insulin Sensitivity of the Domino Children at 5 Years of Age

As shown in Table 2.12, HOMA-IR at 5 years of age was significantly higher in the DHA group (0.80) than in the control group (0.68, p=0.013). While there was no interaction between treatment and sex for HOMA-IR, when the data were analysed separately in males and females HOMA-IR was higher (p=0.0032) in the DHA group in males, but not in females (Table 2.12). Fasting insulin concentrations were also significantly higher (p=0.016) in the DHA group compared with control group overall. Again, while there was no significant interaction between treatment and sex, this effect appeared to be driven mostly by males; fasting insulin levels were significantly higher in the DHA group in males (p=0.014), but were not different in females when the data from the sexes were analysed separately. There was a significant interaction between DHA treatment and sex for fasting glucose levels at 5 years of age, such that fasting glucose levels were significantly higher (p=0.043) in the DHA group in males, but not in females. No child in either treatment group was diagnosed with type 2 diabetes at the time of the 5 year assessment.

Table 2.12: Fasting insulin, glucose and HOMA-IR in the DOMInO children at 5 years of age.

Outcome	*DHA (n=364)*	*Control (n=349)*	*p value*
HOMA-IR: mean (IQ range)			
. Total	0.80 (0.43-1.71)	0.68 (0.38-1.31)	0.01
. Male	0.86 (0.44-1.88)	0.62 (0.35 -1.21)	<0.001
. Female	0.75 (0.43-1.58)	0.74 (0.41-1.41)	0.52
Glucose: mean (SD)			
. Total	4.07 (1.08)	4.02 (1.02)	0.56
. Male	4.26 (1.07)	4.03 (1.00)	0.04
. Female	3.87 (1.04)	4.01 (1.04)	0.29
Insulin: mean (IQ range)			
. Total	4.63 (2.68-9.20)	4.01 (2.38-7.25)	0.02
. Male	4.75 (2.70-9.63)	3.63 (2.22-6.81)	0.01
. Female	4.55 (2.66-8.90)	4.40 (2.56-7.72)	0.37
NO. of type-2 diabetes: N			
. Total	0	0	-
. Male	0	0	-
. Female	0	0	-

HOMA-IR: homeostatic model assessment-insulin resistance

IQ: inter-quartile range

SD: standard deviation

N: number

2.5. Discussion

The results of this Chapter showed that, contrary to expectations, insulin sensitivity was actually significantly lower in children of mothers who had been allocated to a high dose DHA supplement during the second half of pregnancy compared with the control group. This effect appeared to be mainly driven by males in whom both fasting glucose and fasting insulin were significantly increased in the DHA group. The results therefore provide evidence that the maternal DHA supplementation during pregnancy had sex-specific effects on insulin sensitivity in children and may have a negative effect on insulin sensitivity in males.

Given that a number of previous human studies have shown that dietary n-3 LCPUFA supplementation can have beneficial effects on insulin sensitivity[197-199], the apparently negative effect of maternal DHA supplementation on insulin sensitivity in the children was unexpected. However, it is important to note that most human studies that have reported improvements in insulin sensitivity have been conducted in overweight, diabetic and/or elderly adults, and therefore may not be representative of children or infants. In addition, previous studies have had small sample sizes (between 6 and 63 participants) and were probably underpowered to detect differences in insulin sensitivity between groups. Furthermore, not all studies of n-3 LCPUFA supplementation and insulin sensitivity in humans have produced consistent results. One previous randomised controlled trial, for example, reported that n-3 LCPUFA supplementation (2.7g EPA + DHA for 2 months and then 1.7g/day for 4 months) had no effects on insulin sensitivity in type 2 diabetic patients with hypertriglyceridemia[205]. Importantly, another study actually reported negative effects of n-3 LCPUFA supplementation (4g/day for 6 weeks) on short term

glycaemic control in type 2 diabetic participants aged from 40 to 75 years[206]. Thus, the evidence from previous studies in adults on the effect of increased n-3 LCPUFA on insulin sensitivity, glucose control and the risk of type 2 diabetes is not entirely consistent. Moreover, there are some limitations in these previous studies including small number of participants, lack of representative populations and the fact that the n-3 LCPUFA supplementation was typically applied in conjunction with other diet/lifestyle changes, such as reduced caloric intake.

It is also important to note that this is the first study to test the impact of increased exposure of DHA during development on insulin sensitivity in the children, and it is increasingly clear that the effect of the same treatment on the developing organism often differs considerably from its effects in adults[31]. Despite the evidence suggesting that increased DHA supplementation can have beneficial effects on metabolic health in adults, studies have suggested that DHA can have very different (and potentially negative) effects when applied during development. A study conducted in rats, for example, showed that maternal DHA supplementation (15mg/kg/day) during pregnancy and lactation resulted in increased fat accumulation in both male and female offspring (n=11), particularly in subcutaneous fat depots, when compared with offspring of rats fed on a standard chow containing no DHA (n=10)[31]. Similarly, a human RCT trial reported that providing women with a supplement of 4.5g/day of fish oil (n=72) during lactation was associated with a significantly higher BMI in their children at 2.5 years of age compared to children of mothers in the control group (who received 4.5g/day olive oil during this same period) (n=29)[207]. Another RCT also reported that maternal n-3 LCPUFA supplementation (1.5g/day) during the first 4 months of lactation was associated with

adverse effects on blood pressure, energy intake and physical activity in boys at 7 years of age[27]. These data therefore supports the suggestion that the effect of exposure to n-3 LCPUFA during critical developmental periods is likely to have different effects on later metabolic health in the child than the same intervention in adults.

The results of this study also suggested that males are more susceptible to negative effects of prenatal DHA supplementation on insulin sensitivity than females. This is consistent with the results of previous studies which have shown that males are more susceptible to the effect of early life nutritional exposure (e.g. under-nutrition, low protein) on insulin sensitivity than females[79,208]. This greater susceptibility of males to the negative effects of prenatal exposures on subsequent insulin sensitivity may be due to sex differences in body composition, glucose and insulin action, and energy balance[209]. For a given BMI, men have higher lean mass and more visceral and hepatic adipose tissue, whereas women have higher levels of total body fat[209]. These differences in adipose tissue distribution contribute to greater insulin sensitivity in women, as visceral and hepatic adiposity is associated with increased insulin resistance[209,210]. In sheep, previous studies have demonstrated that young males are at greater risk than females for the onset of co-morbidities associated with juvenile-onset obesity, due to the sex-specific differences in adipose tissue distribution and function[211]. In humans, differences in skin-fold thicknesses (a measure of subcutaneous body fat) between males and females have also been reported in children aged between 5 to 19 years, with higher values in females compared to males at the same age[212]. It has been suggested that the lower estrogen levels in males compared to females may also play a role in the greater susceptibility of males

to insulin resistance, since this hormone has a favourable effect on insulin and glucose homeostasis, adipose tissue distribution, and pro-inflammatory markers[209]. Therefore, it is possible that estrogen may counter any negative influence of prenatal DHA exposure on subsequent insulin sensitivity in females, but that this is not the case in males[209]. Although not measured in the current study, previous studies have reported that circulating concentrations of adiponectin, an insulin-sensitising hormone, are also significantly higher in females compared with males, and this may also contribute to the reduced susceptibility of the females in this study to the negative effects of prenatal DHA supplementation on insulin sensitivity[209].

To date, the underlying mechanism of how increased DHA exposure during development may impact on later insulin sensitivity in the child is unclear, and further investigation is required. We found that the n-3 LCPUFA status of the children at 5 years of age was similar between treatment groups. Since the measure of fatty acid status in red blood cells, which was used in this study, reflects long-term fatty acids status, this suggests that the long-term intake of n-3 LCPUFA in the period leading up the assessment of HOMA-IR was similar between the treatment groups. This, coupled with the similar baseline characteristics of the DOMInO women and children between the treatment groups, implies that it was exposure to increased DHA *in utero*, rather than any other differences in other intrauterine exposures, postnatal environmental influences or differences in current DHA intake, which was the reason for the difference in HOMA-IR between the groups at 5 years of age. This suggests that exposure to increased DHA *in utero* may result in altered development of the pathways that control insulin signalling/insulin action, and that

this effect persists until at least 5 years of age. This clearly implicates epigenetic changes as driving this phenotype.

There is growing evidence from animal and human studies that epigenetic process play an important role in the pathway through which the prenatal nutritional environment impacts on the susceptibility of the offspring to metabolic diseases in later life[12,33,34]. Studies in animal models have shown that the maternal nutritional environment can induce alterations in the epigenetic status of offspring, particularly in genes that have an important metabolic function, and that these epigenetic modifications are associated with persistent changes in gene expression levels and, consequently, metabolic function[12,95,96,98,102,103]. While data from human studies is more limited, a recent paper by Godfrey and colleagues reported that decreased intake of carbohydrate during pregnancy was associated with increased DNA methylation status *RXRA* in the umbilical cord blood at delivery[105]. The increased methylation status of this gene was also associated with increased body fat mass in offspring at 9 years of age[105]. It therefore appears that epigenetic modifications induced by changes in maternal nutrition are also likely to play a central role in the mechanisms underlying metabolic programming in humans. Hence, it is important to explore the potential for increased DHA exposure during fetal life to induce epigenetic changes in the children and, in particular, to establish whether these changes could contribute to the reduced insulin sensitivity observed in these children at 5 years of age.

This study produced the unexpected result that increased DHA exposure during late gestation appeared to be associated with a decreased insulin sensitivity in children at

5 years of age. However, due to lack of sufficient cohort sizes to establish normative ranges for insulin sensitivity in young children, and lack of adequate longitudinal studies to relate childhood insulin sensitivity to long-term outcomes, standards for insulin resistance in children have not yet been established[213]. Hence, it is difficult to conclude if this increased HOMA-IR in children in the DHA supplemented group is likely to be of any clinical relevance. However, the difference between the groups was quantitatively small, and it is therefore unlikely that this difference would be clinically significant. However, since the development of type 2 diabetes typically occurs later in life, it will be important to follow up these children at older ages to see if this small but significant difference in insulin sensitivity at 5 years translates into differences in the number of children who go on to develop type 2 diabetes or other metabolic problems in adolescence and adulthood.

2.5.1. Limitations

The results from this study were secondary outcomes, and not primary outcomes of the DOMInO trial or its subsequent follow up studies. Hence, the results from this study may be a chance finding, and need to be interpreted with caution. Moreover, the difference of HOMA-IR, fasting glucose and fasting insulin between treatment groups were small. While participants were instructed that their child should be fasted for at least 4 hours before the appointment, we were not able to confirm that this had been achieved in all cases, and it would be important to repeat this study in a more controlled clinical environment in order to confirm the findings. In addition, given the large number of appointments that needed to be completed, it was not possible to conduct all appointments at the same time of day. Hence, it is not possible to draw a definitive conclusion from the results of the current study that increased n-3 LCPUFA exposure early in life can decrease insulin sensitivity in humans, and further studies are needed to confirm these findings.

2.5.2. Conclusion

To conclude, maternal DHA supplementation during pregnancy was associated with decreased insulin sensitivity at 5 years of age, an effect which was mainly driven by males. It is unknown if this small but significant change in insulin sensitivity at 5 years will affect the children's future risk of type 2 diabetes, and it will be important to follow up these children later in life in order to determine this. This will provide a clear conclusion about the relationship between supplementation of DHA during pregnancy and the risk of insulin resistance, type 2 diabetes or related metabolic problems later in life. It will also be important to determine the mechanism through which increased DHA exposure in fetal life can affect future insulin resistance, in particular the potential role of epigenetic modifications in this mechanistic pathway.

CHAPTER 3: THE EFFECTS OF MATERNAL N-3 LCPUFA SUPPLEMENTATION ON GLOBAL METHYLATION STATUS IN CHILDREN

3.1. Candidate Contribution

I was responsible for receiving, cataloguing and tracking all the samples collected as part of the 3 and 5 year follow-up of the DOMInO study (715 blood samples). I processed all of the blood samples and developed a barcode database for tracking sample locations. I performed the DNA extractions of the buffy coat for all blood samples collected from the DOMInO children at 5 years of age and all of the quality checks for these DNA samples. I had primary responsibility for sending and tracking the consent forms to DOMInO participants in order to obtain consent to access their child's newborn screening cards (NSCs). Once informed consent was obtained, I was responsible for locating, collecting, cataloguing and storing all of the neonatal samples. I also assisted with the global methylation analyses.

3.2. Introduction

Obesity and type 2 diabetes are major public health issues worldwide, and this has led to a growing interest in identifying strategies to reduce the risk of individuals developing these diseases[1,2]. The evidence linking poor nutritional status *in utero* to an increased risk of obesity and associated metabolic diseases in the offspring has led to suggestions that nutritional interventions that are applied before birth and/or in early infancy are likely to be more effective for improving the long-term metabolic health of future generations than interventions which are applied later in life[2,6-8].

N-3 LCPUFAs, in particular DHA, are known to play a critical role in fetal and infant development[126-128]. Recently, there has been growing interest in the potential benefits of an increased supply of these fatty acids *in utero* for later metabolic health outcomes in the child. However, existing studies, including the results presented in Chapter 2 of this thesis, have provided little evidence to support a beneficial effect of prenatal DHA supplementation on body fat mass, BMI or insulin sensitivity later in childhood[214]. Indeed, the results in Chapter 2 suggest that, at least in males, prenatal DHA supplementation may actually be detrimental for insulin sensitivity in early childhood. However, the potential effects of maternal DHA supplementation on the offspring at a genetic or epigenetic level, and therefore the potential mechanisms underlying the observed effect on insulin sensitivity, or other physiological pathways, are not clearly defined.

Although the mechanisms which link the nutritional environment before birth with metabolic outcomes in the child are not clearly understood; previous animal studies have strongly suggested that the programming of both body fat mass and insulin

sensitivity/insulin resistance is mediated, at least in part, by (semi) permanent epigenetic alterations, such as changes in DNA methylation and modifications to histone proteins[9,215]. These epigenetic changes persist after birth, resulting in permanent changes in gene expression and/or the response of the gene to the environment which, in turn, are associated with alterations in the phenotype[33,34]. There is also emerging evidence that *in utero* exposures, including malnutrition[9], smoking[216,217], toxicants[218] or exposure to specific diets and micronutrients[215] can affect global and/or gene-specific patterns of methylation in infancy and beyond.

While data are limited, previous studies have also provided some evidence that exposure to an increased n-3 LCPUFA supply before birth has the capacity to alter DNA methylation in the infant/child[32,219,220]. In rats, feeding dams a high n-3 LCPUFA diet during pregnancy was associated with a significant increase in DNA methylation levels in the promoter of the fatty acid desaturase (*FADS2*) gene, which encodes an important enzyme in PUFA metabolism, in the liver of the offspring[219]. In another rat study, n-3 LCPUFA supplementation during pregnancy in dams fed on a folic or vitamin B12 deficient diet decreased global DNA methylation level in the liver of the offspring on postnatal day 22[220]. Importantly, and of direct relevance to this study, a recent RCT showed that maternal n-3 LCPUFA supplementation (400mg/day DHA) during pregnancy increased global DNA methylation levels compared to control group (olive oil) in cord blood in children (n=52) whose mothers were smokers, but not in those children whose mothers were non-smokers[32]. This suggested, therefore, that exposure to an increased n-3 LCPUFA supply before birth has the potential to induce methylation changes at multiple sites across the genome, and that these effects may be different in different population subgroups[32].

While these data support the hypothesis that epigenetic modifications may play an important role in mediating the effects of early life nutritional/environmental exposures on outcomes in the child, very few human studies have been conducted to date[215], and those that have had small sample sizes, making it difficult to draw robust conclusions. Therefore, it remains unclear whether a defined nutritional treatment during pregnancy such as n-3 LCPUFA supplementation can influence the epigenetic status of the offspring either in the short-term (at birth) or later in life.

The aim of the following two Chapters, therefore, was to determine the impact of maternal DHA supplementation in pregnancy on (a) global DNA methylation (Chapter 3) and (b) gene-specific DNA methylation (Chapter 4) in blood samples collected from children at birth and at 5 years of age. The global DNA methylation level was measured as hypomethylation in LINE-1. While it is recognised that the epigenetic profile in blood may not necessarily reflect that of metabolic target tissues, in the absence of access to these tissues it nevertheless provides an indication of the extent to which a specific exposures influence the epigenome of the child. A secondary aim of this Chapter (Chapter 3) was to determine whether there were any relationships between global DNA methylation status of the children at either birth and 5 years of age and measures of BMI, body fat mass or insulin sensitivity in early childhood.

3.3. Methods and Materials

3.3.1. Study Population

As described in the previous Chapter, the present project was nested within a follow-up study of the DOMInO trial[128].

3.3.2. Neonatal Sample Collection

3.3.2.1. Obtaining Consent to Access Neonatal Screening Cards (NSCs) from the DOMInO Children

The neonatal side study of the DOMInO trial was approved by the Women's and Children's Health Network Human Research Ethics Committee. A consent pack which included the approved participant information sheet requesting permission to access their NSCs for the purpose of epigenetic tests, consent form and reply paid envelope, was sent to all DOMInO participants who had consented to participate in the 3 and 5 year growth and insulin resistance follow-up and had not subsequently withdrawn from the DOMInO study (n=1536 participants). Of the 1,536 participants who were contacted, a total of 1015 provided consent for us to access their child's NSC.

3.3.2.2. Neonatal Screening Card Retrieval

Once written informed consent was obtained from the participants, a set of unique identifiers for each DOMInO participant who consented (mother's full name and date of birth, child's full name and date of birth and Hospital) were used to search the SA Pathology database in order to retrieve the unique laboratory number for the NSC allocated by SA Pathology at the time of collection. This information was then used to identify the location of the NSC in the SA Pathology storage system. The NSCs for the 1,015 DOMInO participants who provided consent for the neonatal study were stored in 18 separate boxes at an off-site storage facility. The boxes were subsequently delivered to SA Pathology at the Women and Children's Hospital 3 boxes at a time. The unique lab number for each child's NSC was then used to locate the NSCs for each DOMInO participant. The unique identifying information on the

card was then cross-checked against the information from the database in order to confirm that only NSCs from the correct individuals were retrieved.

3.3.2.3.Blood Spot Collection from the Neonatal Screening Cards

One blood spot was selected from the NSC of each participant, and 3×3mm diameter punches were collected from a region of the spot that was fully infiltrated with dried blood using a standard hole punch. The punches were placed into a 2mL Eppendorf safe-lock tube (Eppendorf, Hamburg, Germany) labelled with participant details. Thirty punches were made in a sheet of blank collection paper between each of the samples in order to prevent cross-contamination between NSCs. All samples were stored at room temperature prior to DNA isolation. The procedure followed for the neonatal side study, including obtaining consent and sample retrieval and collection, is summarised in Figure 3.1.

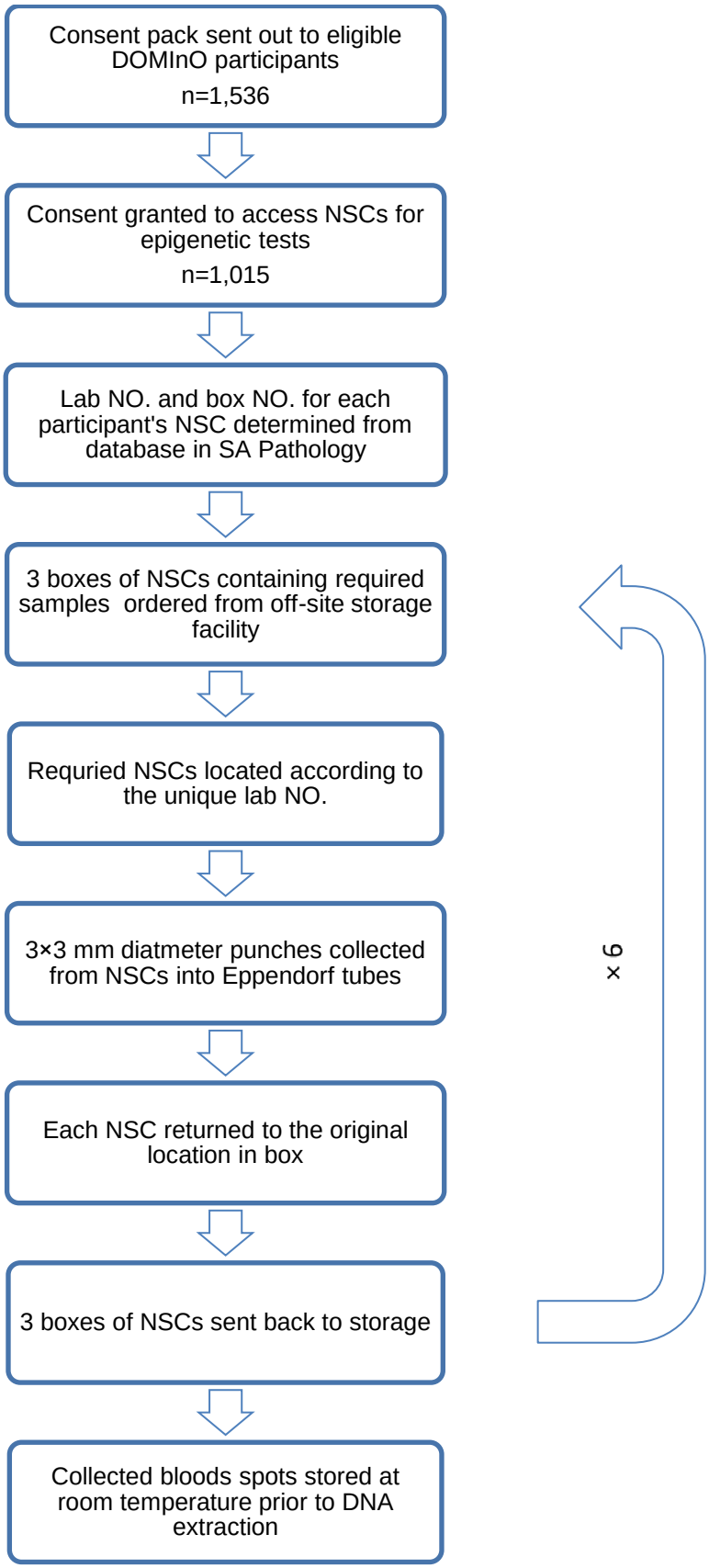

Figure 3.1: Flow chart showing the process of retrieving and collecting bloods spots from NSCs from the DOMInO children.

3.3.3. Blood Sample Collection at 5 Years of Age

The blood samples used in this study were collected from DOMInO children who participated in the 3 and 5 year growth and insulin resistance follow-up of DOMInO and from whom a blood sample was collected at 5 years of age and parental consent provided to undertake (epi)genetic studies.

At the time of the 5 year appointment, blood samples (~5mL into EDTA) were collected from 715 DOMInO children, the primary carer of 669 of whom had provided consent for genetic analysis of the blood samples collected at 5 years of age. After collection, the blood samples were placed in the fridge (4^{o}C), and were then transferred to the laboratory for processing on ice within 24 hours of collection. The blood samples were centrifuged at 3,200rpm for 10 mins to separate RBC, plasma and buffy coat. The buffy coat layer was isolated and transferred to a separate cryovial and stored at -80^{o}C until DNA extraction. The number of samples which were tested for global DNA methylation is shown in Figure 3.2.

3.3.3.1.Anthropometric Measurement

Weight and height were measured in all DOMInO children at the time of the 5 year appointment as described in Chapter 2. BMI was calculated based on the equation: BMI=weight (kg)/height (m)^2. The average of two weight and height measurements was used. Each child's BMI was converted to an age and sex specific z-score using the WHO child growth standards (for children under 61 months old at the time of measurement) or the WHO growth reference 5-19 years (for children 61 months or older at the time of measurement)[221]. Total fat mass and percentage body fat was measured using BIS as described in Chapter 2.

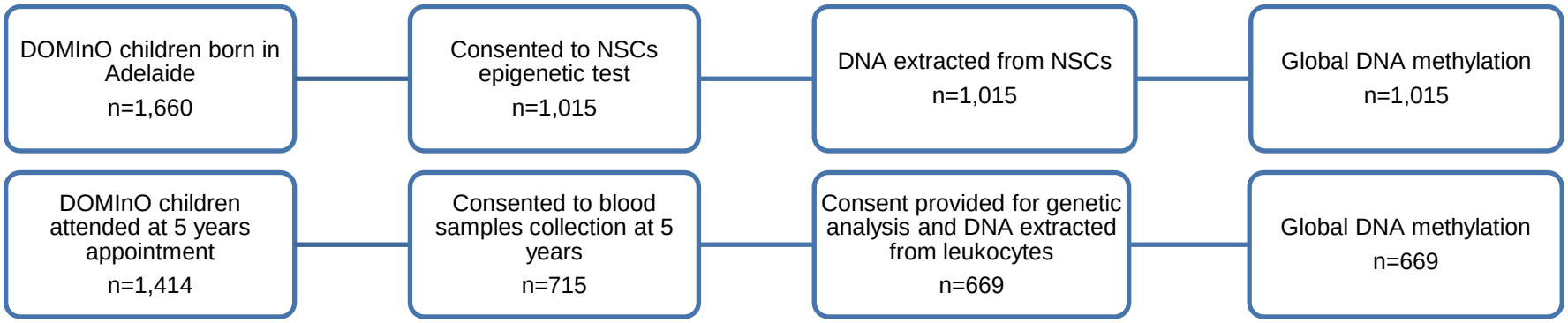

Figure 3.2. Diagram summarising the number of samples obtained from DOMInO children at birth (NSC) and 5 years (blood), and the number of these samples used for global and genome wide DNA methylation analyses at each time point.

3.3.4. DNA Extraction

3.3.4.1. DNA Extraction from the Neonatal Samples

DNA from neonatal samples were extracted using GenSolve technology (IntegenX, Pleasanton, CA America) followed by purification using the QIAamp DNA micro kit (Qiagen, Doncaster, VIC Australia) and an additional ethanol precipitation step. Briefly, the 3×3mm punches from a Guthrie card were incubated for 1 hour at 65°C in 620µL recovery solution A in 1% LiDS, in the presence of protease. After incubation the blood spots were transferred to a spin basket in a new tube and centrifuged for 2 mins at full speed for optimal recovery of the lysate. Subsequently, the spin basket and blood spots were discarded and 20µL of recovery solution B was mixed with the lysate before proceeding with the DNA purification. A volume of 600µL ethanol was added to the lysate and loaded onto a QIAamp micro column. The DNA was purified on the column according to the manufacturer's instructions (Qiagen, Doncaster, VIC Australia) with the modification that the columns and the elution buffer were incubated for 10 mins at 70°C prior to the final centrifugation step.

After elution, the DNA was ethanol precipitated with Glycoblue (Life Technologies, Mulgrave, VIC Australia) and the resulting pellet was dissolved in 60µl AE buffer with 0.01% Triton X100.The quality and quantity of all newborn and 5 year DNA samples was assessed using a NanoDrop spectrophotometer and the Quant-iT Picogreen dsDNA assay (Life Technologies, Mulgrave, VIC Australia). The total amount of DNA obtained from the 3 x 3 mm punches of the neonatal blood spots ranged from ~50 ng to 1500 ng, with an average yield of 450 ng/sample. The

extraction of DNA from neonatal samples was performed by collaborators in CSIRO, Sydney.

3.3.4.2. DNA Extraction from Buffy Coat at 5 Years

DNA from 669 buffy coat samples collected at 5 years of age was extracted using the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Briefly, 200µL of buffy coat was lysed by 20µL Proteinase K (Qiagen) and 200µL Buffer AL. After a 10-min incubation at 56ºC, 200µL 100% ethanol was added into lysates and these were then loaded onto the QIAamp spin column. 500µL of Wash buffers (AW1 and AW2) were used to remove impurities and pure, ready-to-use DNA was then eluted in 200µL Elution Buffer. The quality and concentration of the DNA was determined by measuring the absorbance at 260 and 280nm using a spectrophotometer (NanoDrop Technologies, Wilmington, DE). Ratios of less than 1.8 for either of these measures were considered to be indicative of contamination with proteins or chemicals used in the extraction procedure. No samples had a ratio less than 1.8 and therefore no samples were excluded on this basis. 1µL of DNA solution from every sample was used to check DNA integrity by agarose gel electrophoresis (0.75% w/v agarose gel). All extracted DNA samples were stored at -80ºC prior to epigenetic analysis.

3.3.5. Global DNA Methylation

Measures of global DNA methylation was undertaken for both neonatal (n=1,015) and 5 year DNA samples (n=669). DNA hypomethylation levels in LINE-1 in neonatal and 5 year DNA samples were quantified using the end-specific PCR (ESPCR) assay[36]. In this assay, the relative cutting of the DNA by the methylation-sensitive enzyme HpaII (GeneSearch, Arundel, QLD Australia) is compared with that of the methylation-insensitive enzyme Dra1 (GeneSearch, Arundel, QLD Australia) to give a measure of DNA hypomethylation. The method depends upon the use of 5'-tailed, 3'-blocked oligonucleotides called facilitator oligonucleotides (Foligos). Only cut DNAs with specific matching sequences at their 3' ends can copy the tails of the Foligos and thus become tagged and available for subsequent PCR. Samples were run in triplicate and the DNA hypomethylation levels in the samples were normalised to a reference DNA sample from human blood (Roche Applied Sciences, Dee Why, NSW Australia).

In the ESPCR assay two values, including the level of the hypomethylation in all LINE-1 elements and measure of the amount of DNA in each sample, were calculated. The hypomethylation level of each sample was calculated based on these two values as a ratio of hypomethylation/total DNA amount. One reference sample, consisting of blood from one healthy adult individual was also analysed on each plate. To eliminate plate to plate differences due to slight variations in PCR conditions over time, the plate differences were corrected by dividing the value obtained for each DOMInO sample by the value obtained for the reference sample on the same plate. Hence, the resulting values of each sample are a relative measurement of the degree of LINE-1 hypomethylation in our samples compared to

a standard reference. Thus, a value of 1.5 for the hypomethylation analyses indicates that the LINE-1 hypomethylation level in that sample is 1.5 times higher than the reference. A higher hypomethylation level is indicative of a lower level of global methylation (i.e. the two measures are inversely related).

3.3.6. Statistical Analysis

3.3.6.1. Relationship between DHA Supplementation and Global DNA Methylation

The primary analysis focused on the effect of maternal DHA supplementation in the second half of pregnancy on global DNA methylation at birth and 5 years of age. A linear regression model was fitted with a treatment-by-time interaction term to investigate whether the effect of maternal DHA supplementation at birth differed from the effect of maternal DHA supplementation at 5 years of age. A Generalised Estimating Equation (GEE) with exchangeable correlation was used to account for repeated measures (birth and 5 years). Both unadjusted and adjusted models were fitted, with the adjusted models including centre, parity and sex as covariates.

A secondary question was whether the effect of treatment group was modified by infant sex. To address this question, separate models were fitted for global DNA methylation at birth and at 5 years, with a treatment-by-sex interaction term. Both unadjusted (no covariates other than treatment group and sex) and adjusted models were fitted; the adjusted models included centre and parity as covariates. Differences in global methylation between males and females and between 3 and 5 years were assessed using a Student's unpaired *t*-test and paired *t*-test respectively. $p<0.05$ was considered significant in all analyses.

3.3.6.2. Relationship between Metabolic Health Measures at 5 Years and Global DNA Methylation at Birth and at 5 Years of Age

Secondary analyses were carried out to investigate whether there was any association between global DNA methylation (at birth, or at 5 years) and measures of metabolic

health in the children at 5 years of age (BMI z-score, fat mass percent, insulin, glucose and HOMA-IR). Both insulin and HOMA-IR was log transformed for analysis due to skewed distributions. In addition, interactions between treatment groups (DHA vs control) and sex (male vs female) and relationships between global DNA methylation at birth and at 5 years of age and childhood metabolic health outcomes were also assessed.

The investigation of associations was carried out using Fisher's z-score transformations of the observed correlation coefficients, with z-tests performed to test whether the overall correlation between global DNA methylation (at birth or at 5 years) was significantly different from zero; and also to test whether there was a significant difference in correlation between treatment groups, or between sexes. Analyses of all samples were performed using SAS version 9.3 or later (SAS Institute Inc., Cary, NC USA). A p value of <0.05 was accepted as the level of statistical significance.

3.4. Results

3.4.1. DHA Supplementation and Global DNA Methylation Level at Birth and at 5 Years of Age

There was no difference in the global DNA hypomethylation level between treatment groups either at birth or at 5 years of age (Figure 3.3). There were also no differences in global methylation level either at birth or at 5 years of age between treatment groups when data were analysed separately in males and females (Figure 3.4 and 3.5).

Independent of treatment group, global DNA hypomethylation was higher in females compared to males both at birth and at 5 years of age (p=0.016 at birth, p=0.011 at 5 years). The global DNA hypomethylation level decreased between birth and 5 years, independent of treatment group or sex; indicating that global DNA methylation levels increased from birth to 5 years of age (p<0.0001) (Figure 3.3).

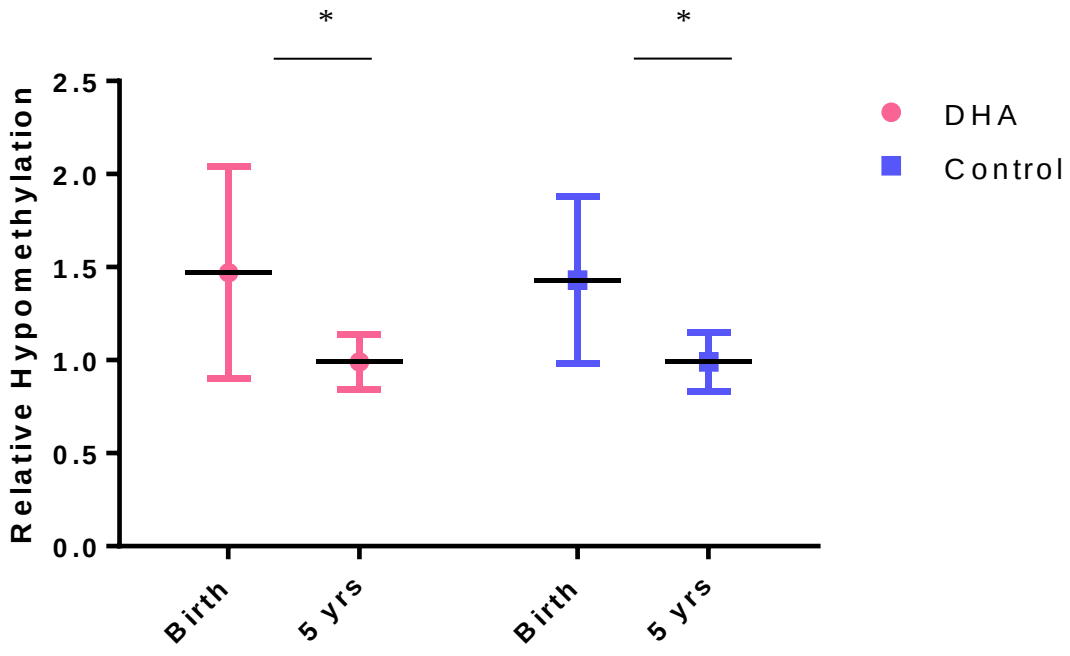

Figure 3.3: The effect of maternal DHA treatment and age on global DNA methylation in children at birth and 5 years of age. Data plotted is mean and SD. * denotes a significant difference between at birth and at 5 years of age within same treatment group (p<0.05). There was no significant difference between treatment groups at either time point.

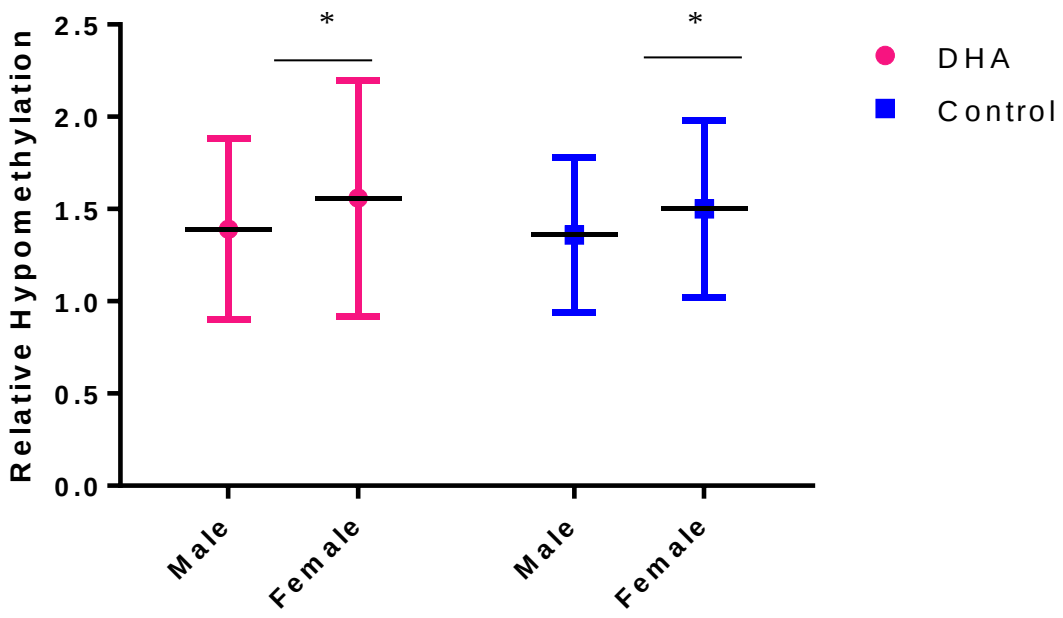

Figure 3.4: The effect of maternal DHA treatment on global DNA methylation in male and female DOMInO children at birth. Data plotted is mean and SD. * denotes a significant difference between male and female children at birth, independent of treatment group (p<0.05). There was no significant difference between treatment groups in either male or female infants.

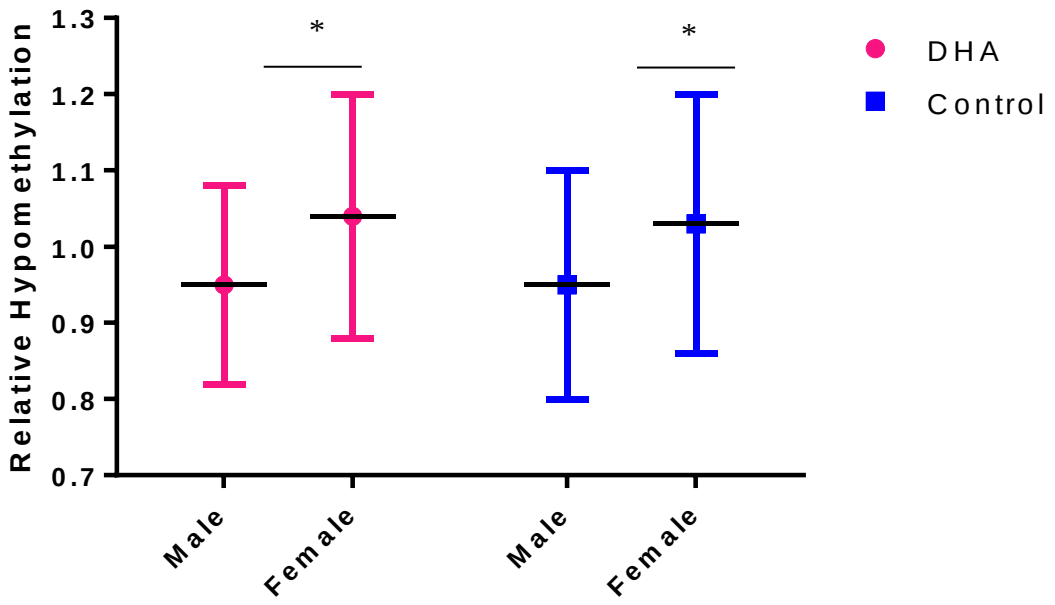

Figure 3.5: The effect of maternal DHA treatment and sex on global DNA methylation in male and female DOMInO children at 5 years of age. Data plotted is mean and SD. * denotes a significant difference between male and female children at 5 years of age, independent of treatment group ($p<0.05$). There was no significant difference between treatment groups in either male or female children at 5 years of age.

3.4.2. Association between Global DNA Methylation Levels at Birth and Measures of Body Composition and Insulin Sensitivity at 5 Years of Age

There was no relationship between global DNA hypomethylation levels at birth and BMI z-score in the sample overall or when data from the DHA and control groups were analysed separately. However, there was a significant difference between the sexes in the relationship between these parameters, such that global DNA hypomethylation at birth and BMI z-score at 5 years of age were positively correlated in males (r=0.009; p=0.044, Table 3.2, Figure 3.5), but not in females. There was also a significant influence of sex on the relationship between global methylation at birth and percentage fat mass at 5 years (p=0.021 for the interaction), however the relationship between these variables was not statistically significant in either males or females when the sexes were analysed separately.

There were no significant correlations between global DNA methylation at birth and fasting glucose or insulin concentrations or HOMA-IR at 5 years of age in either the combined group, or when the DHA and control groups were analysed separately. There were also no significant relationships between global methylation levels at birth and any of these factors when data from male and female children were analysed separately, although there was a trend (r=0.061; p=0.06) towards a positive relationship between global DNA hypomethylation and log insulin concentrations at 5 years of age in males. There was also a trend (r=0.119; p=0.055) towards a positive relationship between global DNA hypomethylation at birth and fasting plasma glucose concentration at 5 years of age in the Control group (Table 3.2).

Table 3.1: Association between global DNA methylation at birth and measures of body composition and metabolic health at 5 years of age

Correlation		*r*	*p value*[1]	*p value for difference*[2]
Methylation and BMI z-score				
. Overall		0.008	0.794	-
. By treatment group	DHA	-0.008	0.861	0.578
	Control	0.028	0.548	
. By sex	Males	0.009	0.044*	0.032*
	Females	-0.048	0.302	
Methylation and fat mass percent				
. Overall		0.057	0.097	-
. By treatment group	DHA	0.062	0.197	0.912
	Control	0.054	0.285	
. By sex	Males	0.085	0.080	0.021
	Females	-0.076	0.128	
Methylation and log insulin				
. Overall		0.064	0.131	-
. By treatment group	DHA	0.064	0.274	0.937
	Control	0.057	0.358	
. By sex	Males	0.110	0.061#	0.329
	Females	0.026	0.673	
Methylation and glucose				
. Overall		0.028	0.515	-
. By treatment group	DHA	-0.026	0.661	0.090
	Control	0.119	0.055#	
. By sex	Males	0.033	0.572	0.841
	Females	0.050	0.417	
Methylation and log HOMA-IR				
. Overall		0.057	0.183	-
. By treatment group	DHA	0.051	0.388	0.917
	Control	0.060	0.339	
. By sex	Males	0.095	0.106	0.461
	Females	0.032	0.609	

1: *p* value for the hypothesis that there is a non-zero overall correlation between the two variables

2: *p* value for the hypothesis that there is a difference in correlation between treatment groups/ sexes

BMI: body mass index

HOMA-IR: homeostatic model assessment-insulin resistance

*: considered as significance when $p<0.05$

#: trend towards significance when $p\leq0.65$

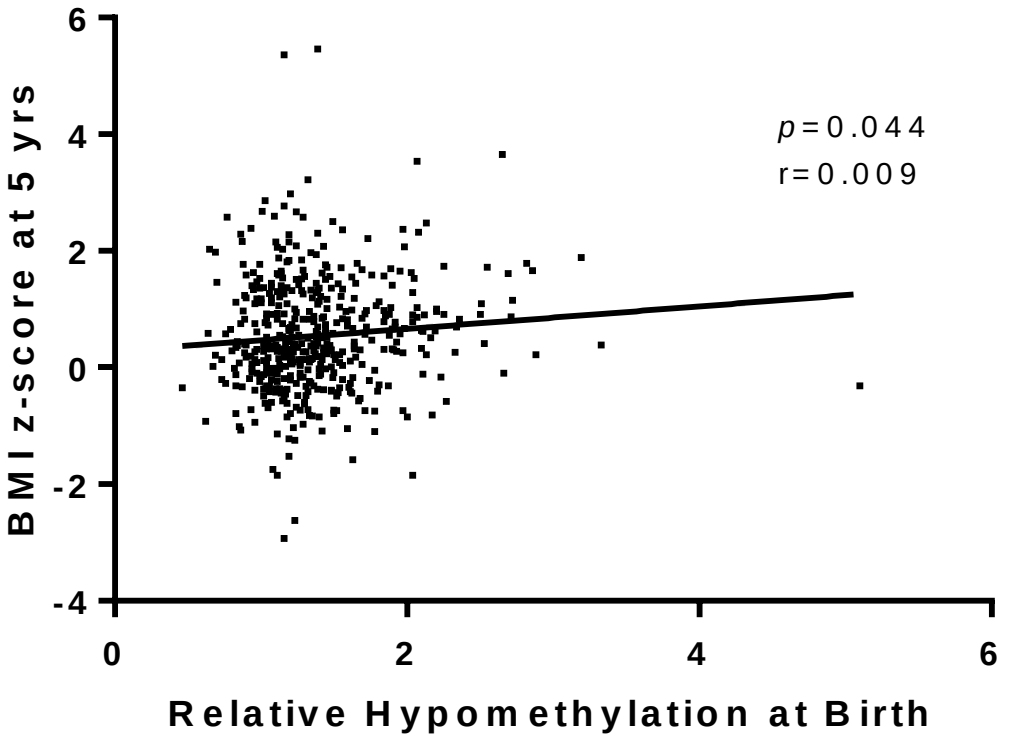

Figure 3.6: Relationship between global DNA methylation at birth and BMI z-score at 5 years of age in male children.

3.4.3. Association between Global DNA Hypomethylation at 5 Years of Age and Measures of Body Composition and Insulin Sensitivity at 5 Years of Age

Global DNA hypomethylation at 5 years of age was positively correlated (r=0.097; p=0.013) with percentage fat mass across the whole study population. While there was no significant difference in this relationship between treatment groups, the positive relationship (r=0.134; p=0.014) was only present in the DHA treatment group when the treatment groups were separated in the analysis (Table 3.3, Figure 3.11).

Plasma glucose concentrations at 5 years of age were negatively correlated (r=-0.082; p=0.036) with global methylation at this age across the whole study population. This relationship was also significantly different between the DHA and control treatment groups (p<0.001), such that the significant negative correlation between glucose and global methylation at 5 years of age was only present in the DHA group (r=-0.205; p<0.001) when the treatment groups were separated in the analysis (Table 3.3, Figure 3.13). Global methylation at 5 years of age also tended to be positively correlated with HOMA-IR in the control group (r=0.107; p=0.055) and in males (r=0.104; p=0.051), but not in the study population overall. There were no significant relationships between global DNA methylation at 5 years and BMI z-score or plasma insulin levels either in the population overall or when data were separated by treatment or sex.

Table 3.2: Association between global methylation and health measurements at 5 years of age

Correlation between		*Correlation*	*p value for corr=0*[1]	*p value for difference*[2]
Methylation and BMI z-score				
. Overall		-0.047	0.233	-
. By treatment group	DHA	-0.052	0.338	0.892
	Control	-0.041	0.461	
. By sex	Males	-0.013	0.811	0.727
	Females	-0.040	0.482	
Methylation and fat mass percent				
. Overall		0.097	0.013*	-
. By treatment group	DHA	0.134	0.014*	0.356
	Control	0.062	0.275	
. By sex	Males	0.048	0.374	0.171
	Females	-0.060	0.297	
Methylation and log insulin				
. Overall		0.050	0.203	-
. By treatment group	DHA	0.031	0.569	0.647
	Control	0.067	0.234	
. By sex	Males	0.090	0.094	0.204
	Females	-0.010	0.866	
Methylation and glucose				
. Overall		-0.082	0.036*	-
. By treatment group	DHA	-0.205	<0.001*	<0.001*
	Control	0.051	0.363	
. By sex	Males	-0.055	0.301	0.962
	Females	-0.059	0.299	
Methylation and log HOMA-IR				
. Overall		0.039	0.320	-
. By treatment group	DHA	-0.032	0.559	0.074
	Control	0.107	0.055#	
. By sex	Males	0.104	0.051#	0.086
	Females	-0.030	0.600	

1: *p* value for the hypothesis that there is a non-zero overall correlation between the two variables

2: *p* value for the hypothesis that there is a difference in correlation between treatment groups/ sexes

*: considered as significance when $p<0.05$

#: trend towards significance when $p\leq0.65$

BMI: body mass index

HOMA-IR: homeostatic model assessment-insulin resistance

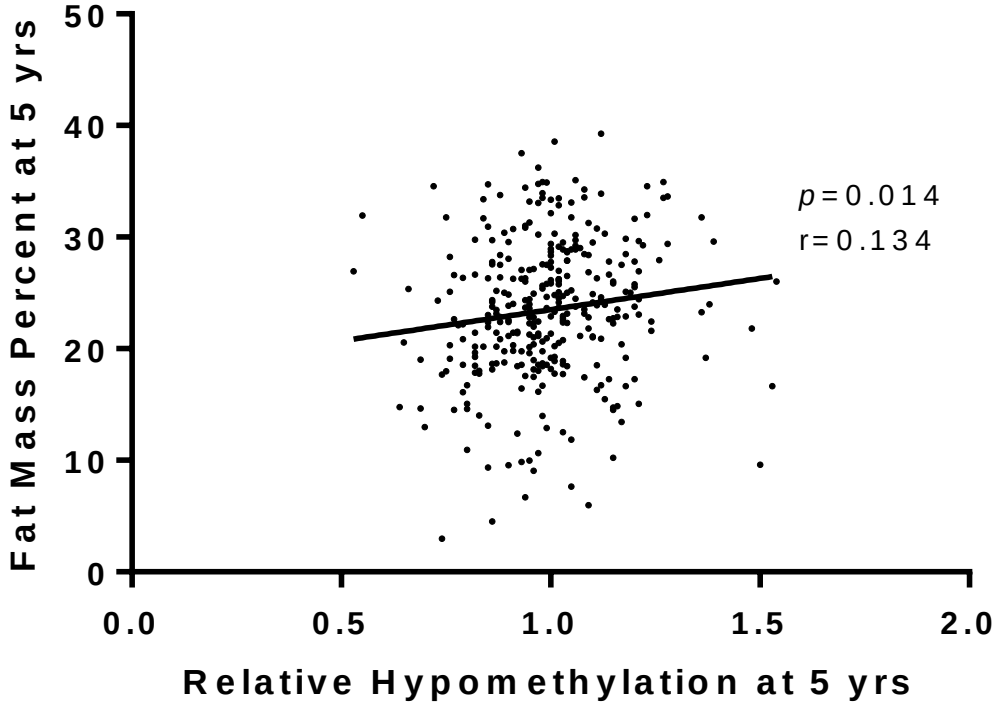

Figure 3.7: Relationship between global DNA hypomethylation at 5 years of age and fat mass percent at 5 years of age in children in the DHA group.

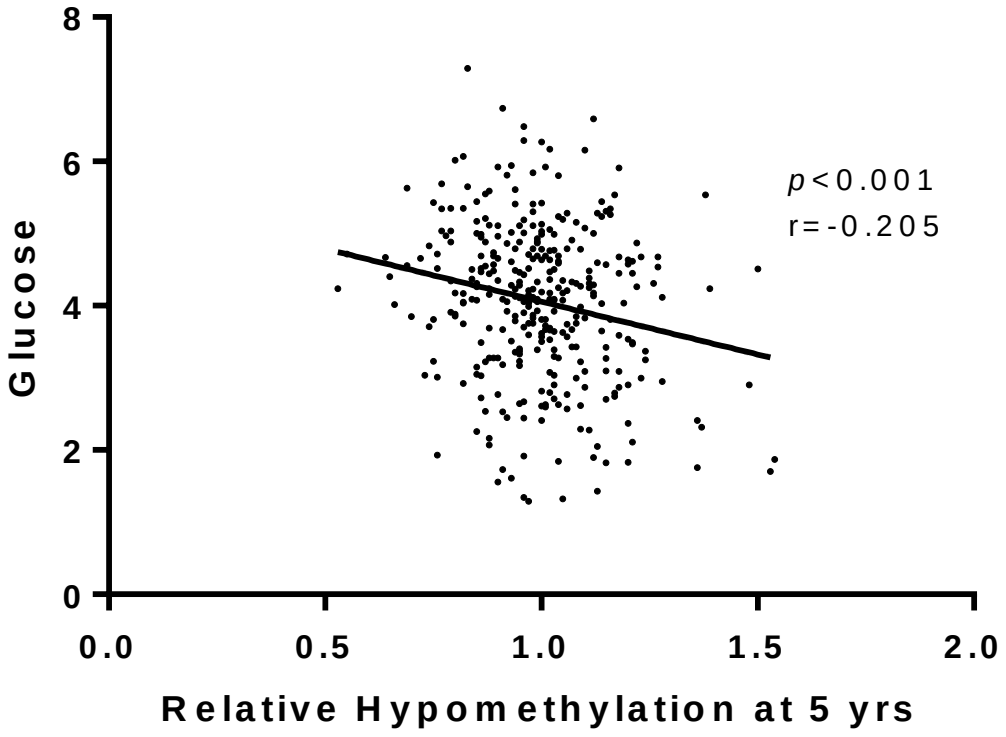

Figure 3.8: Relationship between global DNA hypomethylation at 5 years of age and plasma glucose level at 5 years of age in children in the DHA group.

3.5. Discussion

In this Chapter, I investigated the effects of early life exposure to n-3 LCPUFA supplementation on global DNA methylation levels in the infant at birth and at 5 years of age in a large and well-conducted randomised controlled trial. I found no evidence to support the hypothesis that maternal n-3 LCPUFA supplementation during pregnancy alters global DNA methylation levels in children either at birth or at 5 years of age. I did, however, identify significant relationships between global DNA methylation levels at birth and BMI z-score at 5 years of age in male children, and between global DNA methylation level at 5 years of age and percentage fat mass and plasma glucose levels at this same age in the DHA group, although it is important to note that the correlation coefficients for these relationship were low and therefore the potential biological relevance of these findings is unclear. Overall, the results of this Chapter indicate that exposure to increased n-3 LCPUFA *in utero* has no effect on global DNA methylation level in blood samples collected from children at birth or 5 years of age. In addition, the results provide the first evidence suggesting that global DNA methylation increases from birth to 5 years of age, and is significantly higher in males than in females from the time of birth.

3.5.1. Sex Differences in Global DNA Methylation Level

I found that the global DNA methylation level was lower in females than in males both at birth and at 5 years of age. This finding is consistent with previous studies in separate study populations, which have also reported lower global methylation levels in females compared to males across a wide range of ages and using a range of different techniques for assessing global methylation[147,222,223]. In a combined analysis of 1,465 healthy human subjects (aged from 10 to 100 years) from 5 separate studies, the LINE-1 global DNA methylation level in blood leucocyte which was analysed by using PCR pyrosequencing was lower ($\beta = 0.796$, 95% CI 0.261 to 1.330) in females compared to males[222]. Another study (n=192) which estimated the global methylation level in DNA from whole blood using a luminescence methylation assay, also reported that males (average age was 24.8 years) had significantly ($p<0.0003$) higher global methylation compared to females (average age was 24.3 years)[223]. While the reason for differences in global methylation levels between males and females is unclear; a number of possibilities have been suggested including differences in body composition and/or hormone levels between the sexes[209,211,212].

3.5.2. Age Dependence of Global DNA Methylation Level

The results in this Chapter showed that global DNA hypomethylation level was lower at 5 years of age than in the early neonatal period, indicating a higher level of global DNA methylation level in children at 5 years of age compared to at birth, independent of treatment group or sex. These data therefore suggest that increasing age, at least in the first few years of life, is associated with an increase in the global DNA methylation levels in blood. This is different from the results of several previous studies in both humans and animal models, which have demonstrated that aging is associated with a decrease, rather than an increase, in global DNA methylation. For example, one study which compared the global DNA methylation level in leukocytes in 126 elderly adults (aged from 60 to 88) with 33 healthy younger individuals (aged from 18 to 35) reported that young healthy people had significantly higher levels of (p=0.047) global DNA methylation in their leukocytes, as assessed using an ELISA-based method[224]. Similarly, another study which investigated age-dependent variation of global DNA methylation in human peripheral blood leukocytes, measured by HPLC, showed that the older individuals (average age 65.9 years, n=22) had lower global methylation level compared with younger adults (average age 19.3, n=21)[225]. While our finding is opposite to these previous studies, the majority of these previous studies have not assessed global methylation in the same individuals at different ages. Since epigenetic status can be affected by environment, the different environmental and lifestyle factors to which separate individuals are exposed during their lifetime, independent of the aging process, have the potential to affect the global DNA methylation level. Therefore it is not possible to conclude with certainty from these studies that global methylation in the same individual decreases over time[33,35].

In addition, few studies have assessed changes in global methylation as early in childhood as reported in this Chapter (5 years). Thus, another possibility is that global DNA methylation undergoes an initial increase from birth to early childhood, followed by a decline between later childhood/adolescence and through adulthood. In support of this, Martino and colleagues reported that most (approximately 90% of differentially methylated regions) of the age-associated changes involved an increase in DNA methylation from at birth to 18 months of age in matched samples of 10 monozygotic twin pairs and 5 dizygotic twin pairs using Infinium HumanMethylation450 BeadChip data acquisition and processing[226]. Their results indicated a trend towards increased methylation with age in all regions of the genome in the first 18 months of life, which is consistent with our finding[226]. Thus, the study described in this Chapter, which included a large number of participants and is one of the first to evaluate differences in global DNA methylation level at birth and at 5 years in the same individuals, supports the suggestion that age-related changes in global methylation occur across the first few years of life, and from the time of birth, in humans.

It is also important to note, however, that the DNA samples used in our study at birth and at 5 years were extracted from two different sources of material (dried blood spots at birth and buffy coat at 5 years). A human study which examined the methylation of four differentially methylated regions associated with the *IGF2/H19* locus in multiple birth tissues derived from 91 twin pairs has indicated that considerable variation in DNA methylation was observed between buccal epithelial cells, cord blood-derived mononuclear cells and granulocytes and umbilical vein endothelial cells[227]. It is therefore possible that part of the reason for the age-related

differences in global methylation observed in this study was the use of different sample types at the two time points assessed.

3.5.3. Effect of Prenatal Nutritional Environment on Global DNA Methylation Level

The results presented in this Chapter showed that there was no difference in global methylation level between treatment groups at either birth or at 5 years of age. These results suggest that maternal n-3 LCPUFA supplementation during pregnancy from 20 weeks of gestation until delivery has no significant impact on the global DNA methylation level in peripheral blood in their children either immediately following the intervention (i.e. at birth) or later in childhood.

While limited in number, there are a few previous studies which have investigated the effect of increased exposure to n-3 LCPUFA prenatally on global methylation levels at or after birth, however the dosing and timing of the n-3 LCPUFA supplementation has varied and the studies have produced mixed results. A study in Wistar rats investigated the impact of maternal supplementation with fish oil (1g/kg body weight daily) during pregnancy and lactation on the global DNA methylation level in soleus skeletal muscle and liver using the Imprint Methylated DNA Quantification Kit in both 1st generation and 2nd generation offspring[228], and reported that fish oil supplementation significantly decreased global DNA methylation level in liver, but not in soleus skeletal muscle, but only in 2nd generation offspring[228]. The results of this study are important, since they suggest that the effect of increased prenatal n-3 LCPUFA exposure on global DNA methylation level is tissue specific and may not appear in offspring until the F2 generation.

There is only one previous human study which has investigated the effect of n-3 LCPUFA supplementation during pregnancy on global DNA methylation level in the children[32]. This study assessed global methylation level in umbilical cord blood samples from infants (n=237) whose mothers were supplemented with 400mg algal DHA or a placebo daily from 18-22 gestational week till birth by pyrosequencing[32]. The results of this study found no effect of DHA supplementation during pregnancy on global methylation in the cord blood, consistent with the results of the present study. However, when they split the population into smokers and non-smokers, they found a trend towards increased (p=0.06) global methylation level in the DHA-supplemented group in children whose mothers were smokers, but not in non-smokers[32]. In our study, there were not enough smokers to investigate the effect of DHA supplementation on global methylation within subgroups of smokers and non-smokers. The results presented in this Chapter suggest that prenatal exposure to increased n-3 LCPUFA has no effect on the global methylation level in children at birth overall in the general population, which is consistent with the results of the only other human study in this area. It is possible, however, that different subgroups may respond differently and this will be important to investigate in future studies. Moreover, given the limited number of human studies which have investigated the effect of prenatal n-3 LCPUFA exposure on global methylation, further studies are needed to confirm our findings.

3.5.4. Global Methylation Level at Birth and BMI in Childhood

The finding in this Chapter that there was a positive association between global methylation level at birth and BMI z-score at 5 years of age in males raises the intriguing possibility that global methylation levels at birth may be predictive of later BMI. However, the correlation between BMI z-score at 5 years of age and global DNA methylation level at birth was extremely low, and is therefore unlikely to be of biological or clinical relevance. Furthermore, the capacity of global methylation levels at birth to be of any clinical value in predicting BMI z-score later in childhood is likely to be limited. Nevertheless, this is the first study to identify this relationship and determining whether it can be reproduced in other, and potentially larger, study populations would be of interest. Some studies have determined correlations between BMI and the global methylation level in peripheral blood leukocytes in adults; however, the results have been inconsistent. The majority of these studies, however, have not identified any significant correlation between BMI and global methylation levels[143,222,229]. Zhang and colleagues reported that there was no correlation between BMI and global methylation level in peripheral blood samples in 161 cancer-free subjects aged from 45 to 75 years[229]. Similarly, a large study combining individual data from 1,254 healthy subjects (aged from 19 to 100 years) from 4 studies also failed to identify any correlation between BMI and global methylation level in blood samples[222], and a smaller study of 228 individuals aged from 49 to 51 years also came to a similar conclusion[143].

There are a few studies, however, which have reported an inverse relationship between global methylation level in blood and BMI in adulthood, which this is in the opposite direction to that reported in this Chapter[230]. In one study, it was reported

that women were significantly more likely to have BMI more than 25kg/m^2 when LINE-1 methylation level was less than 62% in peripheral blood (odds ratio of BMI ≥25 vs <25kg/m^2 was 1.89, 95% CI was 1.21 to 2.94, p=0.002)[230]. Another study which examined the association between lifestyle factors and global genomic methylation level in white blood cell DNA from 165 cancer-free subjects (aged from 18 to 78 years) also reported that individuals with a BMI ≥25kg/m^2 had a significantly lower level of LINE-1 methylation than those with BMI <25kg/m^2 (p=0.03)[231]. There is also one previous adult study which identified a significant association between LINE-1 methylation and markers of metabolic and cardiovascular diseases. In this study, which included 355 adult Samoans (88 men and 267 women), the researchers used quantitative bisulphite pyrosequencing to determine global methylation in DNA from peripheral blood samples. While these authors also found a positive association between BMI and LINE-1 methylation, as in this Chapter, they found that the relationship was only present in females, and not males as I did (p=0.03)[232]; however, this may have been due to the low number of male subjects or different methodology used to assess global methylation than in this Chapter[232]. Hence, based on these previous studies and low correlation coefficient between global DNA methylation level at birth and BMI-z score at 5 years of age in our study, there is currently insufficient data to draw any robust conclusions about the association between/global DNA methylation in human blood and BMI/metabolic health.

3.5.5. Association between Global Methylation Level and Metabolic Health

While I did not find any effects of maternal n-3 LCPUFA supplementation on global methylation levels in the children either at birth or at 5 years of age, the relationships between global methylation and both percentage body fat mass percent and plasma glucose levels in the children at 5 years appeared to be altered by prenatal n-3 LCPUFA supplementation. Thus, these relationships were significant in the DHA group, but not the control group, when the treatment groups were analysed separately. This is the first study to show that n-3 LCPUFA supplementation prenatally could potentially alter the relationship between global methylation and measures of metabolic health later in childhood. The mechanisms through which this could operate, and the potential clinical significance of this finding, however, remain unclear.

Global methylation level is considered as a hallmark of cancer because of its correlation with increased mutation events and chromosomal instability in cancer tissue[233]. However, the correlation of global methylation level and other diseases, including obesity and type 2 diabetes, is less well defined. A human study (n=738) which explored the association of global leukocyte DNA methylation levels with various features of the metabolic syndrome, including fasting glucose, high-density lipoprotein cholesterol, triglycerides, blood pressure and waist circumference, reported that a lower level of global methylation in leukocyte DNA was correlated with increased fasting plasma glucose (p=0.004) and high-density lipoprotein cholesterol (p=0.004)[234]. In this same study, it was also reported that individuals (aged 50 to 87 years) with type 2 diabetes or impaired glucose metabolism had lower global DNA methylation levels compared to individuals with normal blood glucose

level (p=0.004)[234]. The results presented in this Chapter were opposite to those of this previous study, since they identified a negative relationship between global DNA hypomethylation level and fasting plasma glucose level (indicative of a positive relationship with global DNA methylation level) and a positive relationship between global DNA hypomethylation level and fat mass percent at 5 years of age (indicative of a negative relationship with global DNA methylation level), but only in children whose mothers were supplemented with DHA in the second half of pregnancy. Although we reported opposite results to the previous study, the population used in our study were much younger (average about 5 years of age) compared to the previous study (average about 69 years of age). In addition, the previous study used different sample sources (DNA extracted from peripheral leukocytes vs Buffy Coat) and different method of determining DNA methylation (liquid chromatography tandem mass spectrometry vs end-specific PCR assay), all of which may have contributed to the disparate findings.

The global DNA methylation level assessed in this study does not provide information on the specific genes/regions of the genome in which methylation is altered. Although the correlation coefficient (r=0.013 for global DNA hypomethylation vs fat mass percent, r=0.036 for global DNA hypomethylation vs glucose) was very low for both relationships, they presence of these relationships nevertheless raises the possibility that prenatal DHA supplementation could influence DNA methylation of specific genes that are linked to body fat mass and insulin sensitivity and that these changes persist until 5 years of age.

3.5.6. Limitations

While the results in this Chapter suggest that prenatal n-3 LCPUFA supplementation does not influence global DNA methylation level at either birth or 5 years of age, it is important to note that global DNA methylation provides a sum total of methylation changes across the whole genome, rather than at specific genes or regions. Therefore, it is still possible that DHA induces methylation changes in individual genes/regions, but that the number of genes/regions whose methylation level was increased was balanced by those in which it was decreased. Hence, further genome-wide methylation data are needed in order to look at whether prenatal DHA can induce methylation changes in specific genes or regions of the genome.

Human studies have suggested that global DNA methylation patterns can be tissue-specific[227]. The DNA used in this study was extracted from peripheral blood, and it isn't clear whether the methylation levels in blood reflects those in target tissues which are responsible for regulating whole-body insulin sensitivity (e.g. adipose tissue, pancreas or muscle). As indicated above, the different source of DNA from individuals at birth and at 5 years of age (blood spots vs buffy coat), may at least in part explain the differences we observed in global methylation at the two ages. Another factor which may have influenced the findings was that the NSCs from which blood spots were obtained were not stored separately, meaning that there was potential for contact of dried blood samples from different subjects and therefore DNA cross-contamination during storage. It is also important to note that global methylation in each individual at each time point was only assessed in a single sample. While this is a common approach in human epigenetic studies, particularly where there are limited opportunities for sample collection, as is the case in infants; it

is nevertheless possible that the global methylation on the day of sample collection was not fully representative of the global methylation of the individual over a longer time period.

3.5.7. Conclusion

In conclusion, the results of this Chapter suggest that global methylation level is age dependent and sex specific. The results further suggest that maternal n-3 LCPUFA supplementation does not significantly alter global methylation levels either at birth or 5 years of age in either male or female children in blood, although it remains possible that different effects would have been observed had we been able to access metabolic target tissues, in particular the skeletal muscle, adipose tissue, liver and pancreas. Moreover, the results of this Chapter demonstrated that global methylation level in peripheral blood was correlated with fasting plasma glucose concentration and fat mass percent at 5 years of age only in children whose mothers were supplemented with n-3 LCPUFA during the second half of pregnancy. This raises the possibility that prenatal DHA supplementation might potentially influence methylation in specific genes that are linked to fat deposition and insulin sensitivity and that these changes persist until 5 years of age, however the very low correlation coefficients for these relationships raises questions about the clinical relevance of the relationships. While global methylation levels in blood does not appear to be influenced by prenatal DHA treatment, there is a need for further studies to investigate whether there are epigenetic changes in specific loci or regions related to body fat mass and glucose metabolism induced by increased exposure to n-3 LCPUFA before birth.

CHAPTER 4: THE EFFECTS OF MATERNAL N-3 LCPUFA SUPPLEMENTATION ON GENOME-WIDE DNA METHYLATION STATUS IN CHILDREN

4.1. Candidate Contribution

I was responsible for tracking all the samples collected as part of this clinical trial and undertook all the sample processing and cataloguing. I performed the DNA extractions for all the blood samples taken at 5 years of age and undertook the associated quality control checks. I played the principal role in the process of sending and receiving consent for collecting neonatal samples from the neonatal screening cards (NSCs). I was also responsible for locating, collecting, cataloguing and storing all of the neonatal samples, and for preparing DNA to be shipped to the external analysis site. I was also involved in discussions with bioinformaticians related to the analysis of the DNA methylation data and undertook all of the PANTHER analysis of the differentially methylated regions and interpretation of these results.

4.2. Introduction

It is now well established that the nutritional environment experienced before birth and/or in early infancy is an important determinant of an individual's risk of developing obesity and metabolic diseases, including type 2 diabetes, in child and adulthood[5,77,79,84,208,235,236]. Although the mechanisms underlying this association are not fully understood, there is increasing evidence that the prenatal environment can alter the epigenome and that this plays an important role in the early origins of obesity and poor metabolic health[9,32,215]. Studies of adult individuals whose mothers were exposed to an acute period of famine (the Dutch Huger Winter) during pregnancy provided the first human evidence to support the importance of the epigenome in metabolic programming. These studies showed that the increased risk of obesity and type 2 diabetes in adult individuals exposed to the famine *in utero* occurred in conjunction with altered DNA methylation of the *IGF2* gene[167,237]. This, together with extensive studies in animal models, has led to the hypothesis that changes in the nutritional environment during critical periods in development can alter the epigenetic status of specific genes involved in metabolic processes and that this has long-term effects on that individuals risk of obesity and type 2 diabetes later in life[215].

The n-3 LCPUFAs, DHA and EPA, are well known for their role in immune regulation, neurodevelopment and growth through the life-course[111,112,136,238]. More recently, there has been growing interest in the potential role of the n-3 LCPUFAs in metabolic health. The potential for increased dietary n-3 LCPUFA intake to reduce body fat accumulation and improve insulin sensitivity has been demonstrated in a number of randomised controlled trials in adults[18-22,115]. These findings have led to

the suggestion that n-3 LCPUFA supplementation during pregnancy could potentially reduce the risk of developing obesity and type 2 diabetes in children after birth. However, the results of studies which have investigated this have produced inconsistent results, and there is a lack of robust evidence as to whether prenatal DHA supplementation is associated with improved metabolic health in the children[25,119-122]. In Chapter 2 of this thesis, I reported that maternal n-3 LCPUFAs supplementation in the second half of pregnancy was actually associated with decreased insulin sensitivity in children at 5 years of age, however the mechanistic basis of this is not known.

Recently, a number of studies have provided evidence to suggest that n-3 LCPUFA have the capacity to influence epigenetic processes in both developing and adult individuals[239]. In rats, intake of n-3 LCPUFAs during pregnancy was shown to increase DNA methylation levels in the promoter region of the *Fads2* gene in the liver of the offspring, a gene which plays a critical role in PUFA metabolism, which was negatively correlated with *Fads2* mRNA expression[219]. The results of this study suggested that consuming a diet enriched in n-3 LCPUFAs during pregnancy can persistently change epigenetic status in the offspring and result in long-term effects on gene expression and PUFA synthesis[219]. A human study also reported differences in DNA methylation at 27 sites between adult individuals with high and low n-3 PUFA intakes[240]. In another human study, maternal supplementation with n-3 LCPUFAs during the second half of pregnancy altered the global methylation level in cord blood in women who were smokers, but had no effect in non-smokers[32].

In Chapter 3 of this thesis, I reported that maternal n-3 LCPUFA supplementation in the second half of pregnancy did not result in any change in the global methylation level in children at birth and at 5 years of age. However, as discussed in that Chapter, the global methylation level only provides information on the overall methylation status of the genome, and does not indicate if there are alterations in the methylation level of specific genes or regions. In Chapter 3, I also identified significant associations between global DNA methylation levels and the fat mass percent and plasma glucose levels in children at 5 years of age that were only present in the DHA treatment group, but not the control group. This led me to hypothesise that exposure to an increased supply of n-3 LCPUFA *in utero* could potentially alter DNA methylation patterns in some specific genes or regions involved in fat deposition and/or glucose metabolism, and that these changes persist at 5 years of age. However, no previous human studies have determined the effects of increased exposure to n-3 LCPUFA prenatally on DNA methylation in offspring.

Therefore, the aim of this Chapter was to determine the impact of maternal DHA supplementation during the second half of pregnancy on gene/region-specific genome-wide DNA methylation in children at birth and at 5 years of age using DNA samples from the DOMInO RCT.

4.3. Methods and Materials

4.3.1. Study Population

As described in the previous Chapter, the present project is nested within a follow-up study of the DOMInO trial[128], details of which have been provided in Chapter 2.

4.3.2. Neonatal Sample Collection

The same procedure for obtaining consent to access the NSCs for the DOMInO children, locating the NSCs and obtaining the dried blood punches were followed as described in Chapter 3.

4.3.3. Blood Sample Collection at 5 Years of Age

The subset of DOMInO children whose blood samples were used in this study consisted of children from whom blood samples were collected at 5 years of age and who had provided consent for (epi)genetic tests/DNA isolation to be conducted on these blood samples. Details of the procedures followed for sample collection, processing and storage of samples were described in Chapter 3. The number of samples which were tested for genome-wide DNA methylation is shown in Figure 4.1.

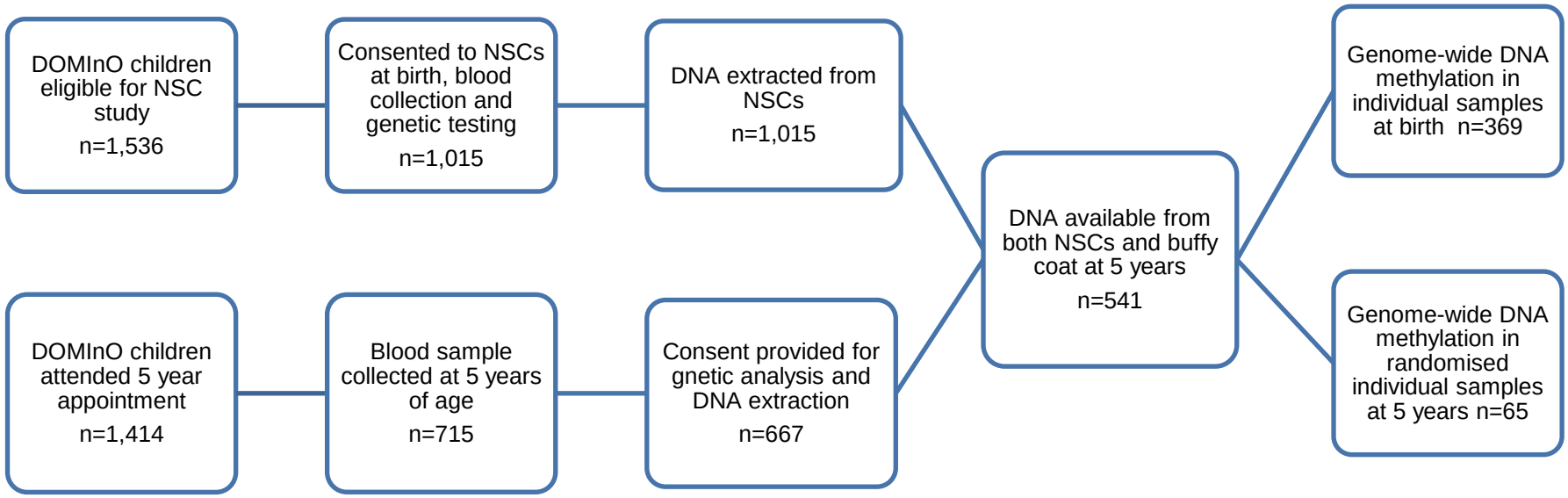

Figure 4.1: Diagram depicting the number of samples from the DOMInO children utilised for genome-wide DNA methylation from DNA extracted from neonatal screening cards (NSCs) at birth and buffy coat samples collected at 5 years of age.

4.3.4. DNA Extraction

4.3.4.1. DNA Extraction of Neonatal Samples

DNA extraction of neonatal samples was conducted as described in Chapter 3. Briefly, DNA from neonatal samples was extracted using GenSolve technology (IntegenX, Pleasanton, CA America) followed by purification using the QIAamp DNA micro kit (Qiagen, Doncaster, VIC Australia) and an additional ethanol precipitation step. The quality and quantity of all samples was assessed using a NanoDrop spectrophotometer and the Quant-iT Picogreen dsDNA assay (Life Technologies, Mulgrave, VIC Australia). The extraction of NSC blood spots was performed by collaborators in CSIRO, Sydney.

4.3.4.2. DNA Extraction from Buffy Coat at 5 Years

The DNA extraction of blood samples collected from 5 year olds was conducted as described in Chapter 3. Briefly, DNA was extracted from the buffy coat fraction using the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The quality and concentration of the DNA was determined by measuring the absorbance at 260 and 280nm (NanoDrop Technologies, Wilmington, DE). 1µl of DNA from each sample was used to confirm DNA integrity by agarose gel electrophoresis (0.75% w/v agarose gel).

4.3.5. Genome-wide DNA Methylation Analysis

Genome-wide DNA methylation was assessed using the Illumina Infinium HumanMethylation450 (450K) BeadChip in DNA samples from a subset of 369 children at birth and 65 children at age 5. The Illumina Infinium HumanMethylation 450 BeadChip is a high-density microarray for quantifying the methylation level of over 450,000 CpG sites within human genome[241]. The 450K platform covers 99% of RefSeq genes with multiple sites in the annotated promoter (1500bp or 200bp upstream of transcriptin start site), 5' UTR, first exon, gene body and 3' UTR. From the CpG context, it covers 96% of Cpg islands with multiple sites in the annotated CpG Islands, shores (regions flanking island) and shelves (regions flanking shores)[241,242]. Hence, the 450K platform is a powerful tool for exploring methylation profile in these annotated regions[241]. The subset of DOMInO children at birth comprised of those for which a sufficient quantity of DNA was obtained from the neonatal screening card for individual analysis (>300ng) and for whom DNA samples were also available at age 5 years. The subset of 65 children at age 5 years was selected at random by an external statistician to ensure an equal distribution of treatment groups and sexes.

All selected DNA samples were submitted to the Australian Genome Research Facility (Parkville, VIC Australia) for bisulfite conversion using the Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA USA). Bisulfite treated DNA was hybridised to the 450K arrays and the arrays were processed following standard protocols. Samples were randomised across arrays according to treatment group, sex and date of birth to minimise the impact of batch effects.Statistical Analysis

Normalisation of the raw intensity data from the 450K array was performed using the *dasen* method from the Bioconductor software package *wateRmelon*[243]. The union of probes located on the sex chromosomes (n=11,648), probes targeting CpGs located 2 or fewer nucleotides from a known Single Nucleotide Polymorphism (SNP) with a minor allele frequency >0.05 (n=29,476), and known cross-hybridising probes (n=30,969)[244] were excluded from the analysis. Probes were also excluded if they failed in one or more samples, based on a detection *p* value of greater than 0.05.

To identify differentially methylated probes in the DHA group compared to the control group, the limma package[245] was used on quantile normalised beta values to compute a moderated t- test. Raw p values were corrected for multiple testing using the Benjamini-Hochberg method and significant differentially methylated probes were identified based on a False Discovery Rate (FDR) *p*-value <0.05. The methylation values are expressed as beta values, on a scale of 0 (unmethylated) to 1 (fully methylated), these can also be interpreted as percentage methylation (1=100%, 0.5=50%, 0=0%).

The methylated regions (DMRs) between the DHA and the control group were identified using the Bioconductor package *DMRcate*[246], which extracts these regions via kernel density modelling. A bandwidth size of 500bp was used, and based on a FDR *p*-value cut-off of <0.05, the most significant DMRs in the genome were agglomerated and extracted. Only regions that constituted of more than 1 probe were selected.

The difference of methylation level in DMRs between two treatment groups is expressed as maximum beta difference. This refers to the maximum beta difference (average beta value for DHA group minus average beta control group) that was calculated for each probe that was found within that DMR. For example, a maximum beta difference of 0.01 indicates a 1% difference in methylation level at that DMR.

Biological pathway analysis of DMRs was assessed using the PANTHER (Protein Analysis Through Evolutionary Relationships) Classification System (version 9.0, release date 20th Jan 2014) which contains 7,180 protein families, divided into 52,768 functionally distinct protein subfamilies.

4.4. Results

4.4.1. Genome-wide DNA Methylation

4.4.1.1. Effect of Prenatal DHA Supplementation on Genome Wide DNA Methylation at Birth

There was no significant difference in the average DNA methylation levels obtained by annotation across all probes on the 450K array between the DHA and control groups at birth (Table 4.1). DNA methylation level in males was higher than in females across all designations.

There were a total of 44 (adjusted p <0.05) DMRs identified between the DHA and control group at birth, 37 of which were associated with a gene and 7 of which were intergenic (Table 4.2). Interestingly, the majority of DMRs showed higher methylation levels in the DHA group compared to the control group, with only 6 DMRs showing lower methylation in the control group. The beta differences (average beta value for DHA group minus average beta value for control group) between groups indicated that the methylation differences between the DHA and control groups were very small, ranging from -5% to 4%. To investigate whether the DMRs clustered in any particular biological pathways, they were analysed using the Protein Analysis Through Evolutionary Relationships (PANTHER) Classification System (Figure 4.2). The results of the PANTHER analysis indicated that the 44 DMRs were mainly clustered in metabolic (14 DMRs), cellular (13 DMRs), developmental (9 DMRs) and immune system (7 DMRs) processes (Figure 4.2).

Table 4.1 Global DNA methylation levels across all probes by genomic location

	DHA group [1]		*Control group*		*Group comparison (p value)*[2]	*Sex comparison (p value)*
	Male	Female	Male	Female		
TSS1500	0.332	0.331	0.332	0.331	0.876	<0.001
TSS200	0.177	0.176	0.178	0.176	0.292	<0.001
5'UTR & 1st Exon	0.298	0.296	0.298	0.296	0.521	<0.001
Body	0.593	0.592	0.593	0.592	0.878	<0.001
3'UTR	0.691	0.689	0.691	0.689	0.682	<0.001
Intergenic	0.564	0.563	0.565	0.563	0.876	<0.001
All (combined)	0.472	0.471	0.472	0.471	0.781	<0.001

[1] mean methylation beta value by genomic location as annotated on the Illumina Infinium 450k arrays, [2] all P values in the table are Benjamini Hochberg adjusted P values

Abbreviations: TSS1500, 1500 base pairs from the transcription start site; TSS200, 200 base pairs from the transcription start site; 5'UTR, 5' Untranslated Region, 3'UTR, 3'Untranslated Region.

Table 4.2: Differentially Methylated Regions (DMRs) between the DHA-supplemented and control group at birth

Genomic range	*Probes*	*Gene symbol*	*Gene name*	*Genomic location*	*Adjusted p value*	*Max beta difference*[*]
chr3:138152837-138153439	11	ESYT3	extended synaptotagmin-like protein 3	TSS1500,TSS200,1stExon,5'UTR	2.63E-08	0.04
chr12:132293329-132293702	5				2.55E-06	0.03
chr15:34610829-34611069	4	SLC12A6	solute carrier family 12 (potassium/chloride transporter), member 6	Body,1stExon,5'UTR,TSS200	1.42E-05	0.01
chr3:42201314-42201898	6	TRAK1	trafficking protein, kinesin binding 1	TSS1500,Body,TSS200,1stExon	6.79E-05	0.02
chr6:166876490-166877038	7	RPS6KA2	ribosomal protein S6 kinase, 90kDa, polypeptide 2	Body	2.88E-04	-0.02
chr10:134221503-134222507	8	PWWP2B	PWWP domain containing 2B	3'UTR,Body	2.96E-04	0.03
chr17:75445905-75446661	7	SEPT9	septin 9	Body,TSS1500,TSS200,1stExon,5'UTR	5.15E-04	0.01
chr8:59058254-59058660	5	FAM110B	family with sequence similarity 110, member B	5'UTR	5.29E-04	0.01
chr7:1882776-1883876	9	MAD1L1	MAD1 mitotic arrest deficient-like 1 (yeast)	Body	5.29E-04	-0.02
chr12:7780736-7781431	6				2.88E-03	0.05
chr3:42306150-42307193	10	CCK	cholecystokinin	5'UTR,1stExon,TSS200,TSS1500	2.92E-03	0.01
chr4:62382932-62383240	4	LPHN3	latrophilin 3	Body	4.19E-03	0.02
chr6:150346721-150347053	10	RAET1L	retinoic acid early transcript 1L	TSS200,TSS1500	4.37E-03	0.01
chr6:31549853-31550241	4	LTB	lymphotoxin beta (TNF superfamily, member 3)	Body,1stExon,TSS200	4.62E-03	0.01
chr6:150038536-150038700	2	LATS1	large tumor suppressor kinase 1	5'UTR	4.86E-03	0.00
chr22:32599511-32599648	3	RFPL2	ret finger protein-like 2	TSS200,5'UTR,TSS1500	8.25E-03	0.04
chr3:71276214-71276336	2	FOXP1	forkhead box P1	5'UTR	9.88E-03	0.02

chr6:1619094-1621093	8				0.010	0.01
chr10:134755862-134756994	9	CFAP46	cilia and flagella associated protein 46	Body,1stExon,TSS200,TSS1500	0.010	0.02
chr3:49236800-49237500	8	CCDC36	coiled-coil domain containing 36	TSS200,5'UTR,1stExon	0.011	0.01
chr19:50861262-50862121	6	NAPSA	napsin A aspartic peptidase	3'UTR,Body	0.012	0.02
chr2:242800973-242801252	3	PDCD1	programmed cell death 1	1stExon,5'UTR,TSS200	0.012	0.01
chr12:130824015-130824831	6	PIWIL1	piwi-like RNA-mediated gene silencing 1	5'UTR	0.014	0.02
chr1:240656217-240656737	6	GREM2	gremlin 2, DAN family BMP antagonist	3'UTR,Body	0.014	0.02
chr4:1107202-1107259	2	RNF212	ring finger protein 212	1stExon,5'UTR	0.014	0.01
chr1:156260918-156261403	6	TMEM79	transmembrane protein 79	Body,3'UTR	0.016	-0.01
chr1:245319261-245319431	2	KIF26B	kinesin family member 26B	Body	0.017	0.00
chr17:80541737-80542118	4	FOXK2	forkhead box K2	Body	0.019	0.02
chr19:53540845-53541287	4				0.021	0.01
chr16:2907859-2908554	6	PRSS22	protease, serine, 22	Body,1stExon,5'UTR,TSS200,TSS1500	0.025	0.01
chr15:93580022-93580327	4				0.029	-0.03
chr10:105210937-105210980	2	CALHM2	calcium homeostasis modulator 2	5'UTR,Body	0.029	0.01
chr8:117950244-117950504	7	AARD	alanine and arginine rich domain containing protein	TSS1500,TSS200,1stExon,5'UTR	0.029	0.01
chr20:13620031-13620048	2	TASP1	taspase, threonine aspartase, 1	TSS1500	0.038	-0.01
chr8:29172906-29172998	3				0.041	0.02
chr2:48844728-48845068	8	GTF2A1L,STON1	general transcription factor IIA, 1-like, stonin 1	TSS1500,Body,TSS200,1stExon,5'UTR	0.045	0.02
chr20:3051954-3052345	9	OXT	oxytocin/neurophysin I	TSS1500,TSS200,1stExon,5	0.045	0.01

			prepropeptide	'UTR		
chr15:45571596-45571636	2				0.045	0.01
chr17:76975944-76976091	4	LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	1stExon,5'UTR,TSS200	0.046	0.01
chr19:57988860-57989134	7	ZNF772	zinc finger protein 772	1stExon,5'UTR,TSS200	0.046	0.01
chr6:30460244-30460322	2	HLA-E	major histocompatibility complex, class I, E	Body	0.046	0.01
chr21:43823604-43823835	4	UBASH3A	ubiquitin associated and SH3 domain containing A	TSS1500,TSS200	0.046	0.01
chr6:29910755-29910776	2	HLA-A	major histocompatibility complex, class I, A	Body	0.048	-0.01
chr8:141359539-141359786	4	TRAPPC9	trafficking protein particle complex 9	Body	0.049	0.02

*: The beta difference (average beta value for DHA group minus average beta for control group) is calculated for each probe, and the maximum beta difference shows the highest beta difference that was found within that DMR.

TSS1500: 1500 base pairs from the transcription start site.

TSS200: 200 base pairs from the transcription start site.

5'UTR: 5' untranslated region.

3'UTR: 3' untranslated region.

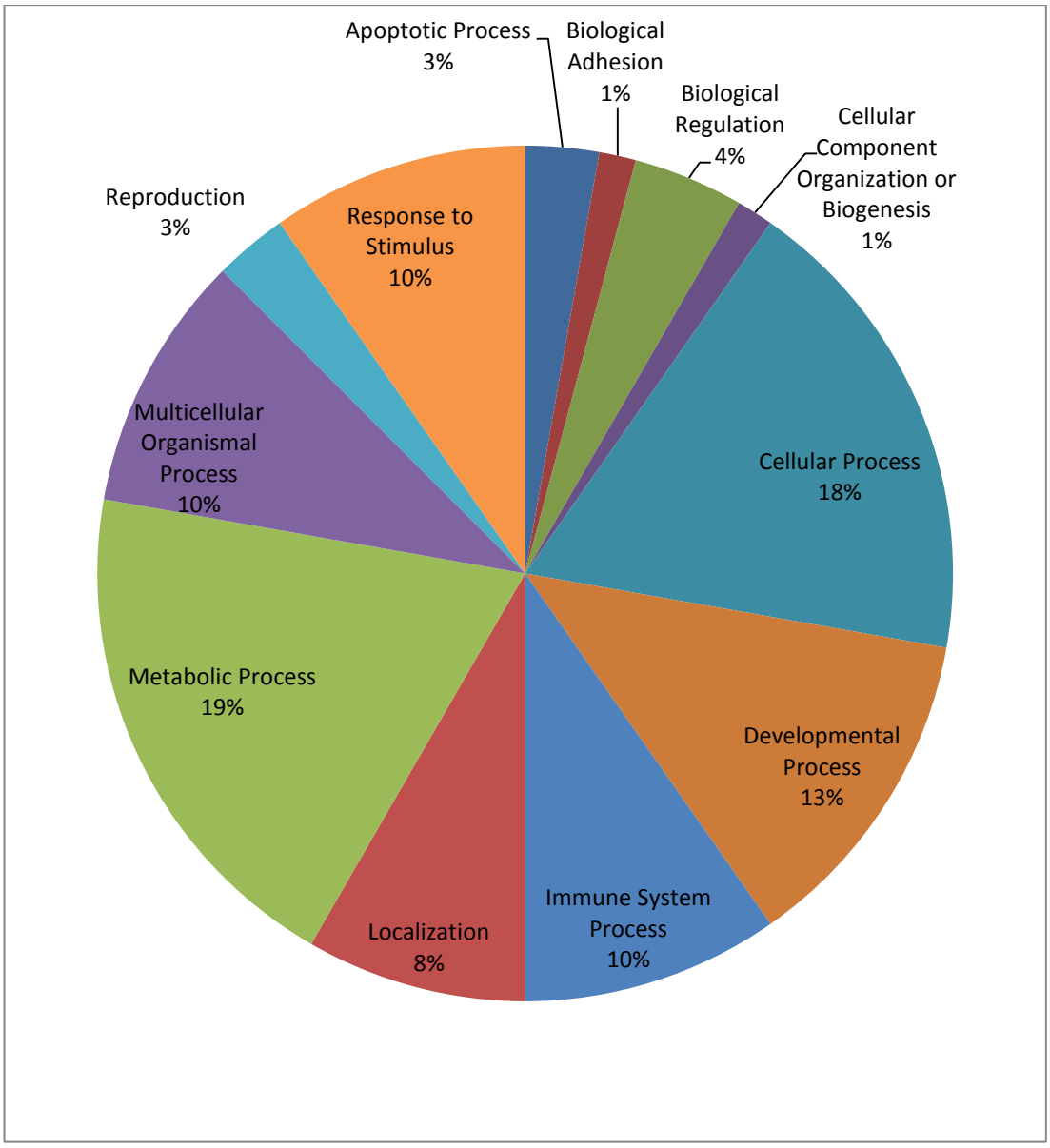

Figure 4.2: Pie chart analysis results showing the biological processes associated with genes identified as differentially methylated between DHA and control group at birth as determined using the PANTHER Classification System.

4.4.1.2.Effect of Prenatal DHA Supplementation on Genome Wide DNA Methylation at 5 Years

Consistent with data at birth, there was no significant difference of mean DNA methylation levels by annotation across all probes on the 450K array between DHA and control group at birth. DNA methylation level in males was higher than in females across all designations

At 5 years of age, a total of 30 (p <0.05) DMRs were identified between the DHA and control groups. 22 of these DMRs were associated with a gene and 8 DMRs were intergenic (Table 4.3). Interestingly, and similar to the findings at birth, the majority of DMRs showed higher methylation levels in the DHA group compared to the control group, with only 5 DMRs showing lower methylation in the control group. Moreover, methylation differences between the DHA group and control group were small, which is also consistent to the findings at birth. Biological process analysis from PANTHER showed that these 30 DMRs were mainly clustered in metabolic (14 DMRs), cellular (8 DMRs) and developmental (8 DMRs) processes, which was a similar pattern to that observed in the neonatal samples. In addition, one gene (*MD1L1*) was differentially methylated at both birth and 5 years of age; however, the direction of change in *MD1L1* was different at 5 years (max beta difference: 0.06) of age compared to at birth (max beta difference: -0.02).

Table 4.3: DMRs between the DHA-supplemented and control group at 5 years of age

Genomic range	*No. probes*	*Gene symbol*	*Gene name*	*Genomic location*	*Adjusted p*	*Max beta difference*[*]
chr1:75198211-75199496	12	TYW3, CRYZ	tRNA-yW synthesizing protein 3 homolog, crystallin	TSS1500,5'UTR,TSS200,1stExon, Body	1.9E-09	0.05
chr1:152161237-152162507	8				2.7E-08	0.09
chr19:11784246-11785337	15	ZNF833	zinc finger protein 833, pseudogene	TSS1500,TSS200,Body	3.4E-08	0.05
chr3:39543515-39544326	6	MOBP	myelin-associated oligodendrocyte basic protein	5'UTR,Body	6.3E-06	0.07
chr8:38757532-38757859	5	PLEKHA2	pleckstrin homology domain containing, family A member 2	TSS1500	1.1E-05	0.08
chr1:92012148-92012736	6				5.9E-05	0.05
chr3:46758813-46759698	10	PRSS50	protease, serine, 50	Body,1stExon,5'UTR,TSS200,TSS1500	6.1E-05	0.06
chr1:42611386-42612518	5				1.1E-04	0.07
chr15:74218418-74219307	12	LOXL1	lysyl oxidase-like 1	TSS1500,TSS200,5'UTR,1stExon	1.6E-04	0.03
chr10:134938144-134938528	3	GPR123	G protein-coupled receptor 123	Body	3.5E-04	0.05
chr19:46526100-46527410	11	PGLYRP1	peptidoglycan recognition protein 1	1stExon,5'UTR,TSS200,TSS1500	4.5E-04	0.03
chr11:637885-638076	2	DRD4	dopamine receptor D4	Body	9.9E-04	-0.05
chr1:207842303-207843560	6	CR1L	complement component (3b/4b) receptor 1-like	Body	1.1E-03	0.05
chr6:41376295-41376604	2				1.7E-03	-0.04

chr1:110254662-110255096	10	GSTM5	glutathione S-transferase mu 5	TSS1500,TSS200,1stExon,5'UTR, Body	2.1E-03	0.10
chr17:17109239-17110207	13	PLD6	phospholipase D family, member 6	1stExon,5'UTR,TSS200,TSS1500	3.5E-03	0.07
chr16:88589918-88590467	6	ZFPM1	zinc finger protein, FOG family member 1	Body	0.010	0.04
chr6:164506861-164507305	6				0.011	0.05
chr17:19628012-19628421	4				0.012	0.05
chr6:169977394-169977608	2	WDR27	WD repeat domain 27	Body	0.013	0.05
chr3:182817190-182817626	11	MCCC1	methylcrotonoyl-CoA carboxylase 1 (alpha)	1stExon,5'UTR,TSS200,TSS1500	0.016	0.02
chr17:78912765-78913111	2	RPTOR	regulatory associated protein of MTOR, complex 1	Body	0.016	-0.12
chr6:31695415-31695599	3	DDAH2	dimethylarginine dimethylaminohydrolase 2	Body	0.016	0.04
chr6:30419491-30419612	6				0.019	-0.05
chr8:144654887-144655030	4	C8orf73/ MROH6	maestro heat-like repeat family member 6	1stExon,5'UTR,TSS200	0.023	0.04
chr15:26874098-26874515	4	GABRB3	gamma-aminobutyric acid (GABA) A receptor, beta 3	Body	0.024	0.04
chr10:105420747-105421249	4	SH3PXD 2A	SH3 and PX domains 2A	Body	0.028	0.04
chr19:47507399-47507691	4	GRLF1/A RHGAP3 5	Rho GTPase activating protein 35	3'UTR	0.030	0.06
chr7:1915268-1915363	3	MAD1L1	MAD1 mitotic arrest deficient-like 1 (yeast)	Body	0.037	0.06
chr6:7468673-7468973	4				0.046	-0.04

*: The beta difference (average beta value for DHA group minus average beta for control group) is calculated for each probe, and the maximum beta difference shows the highest beta difference that was found within that DMR.

TSS1500: 1500 base pairs from the transcription start site.
TSS200: 200 base pairs from the transcription start site.
5’UTR: 5’ untranslated region.
3’UTR: 3’ untranslated region.

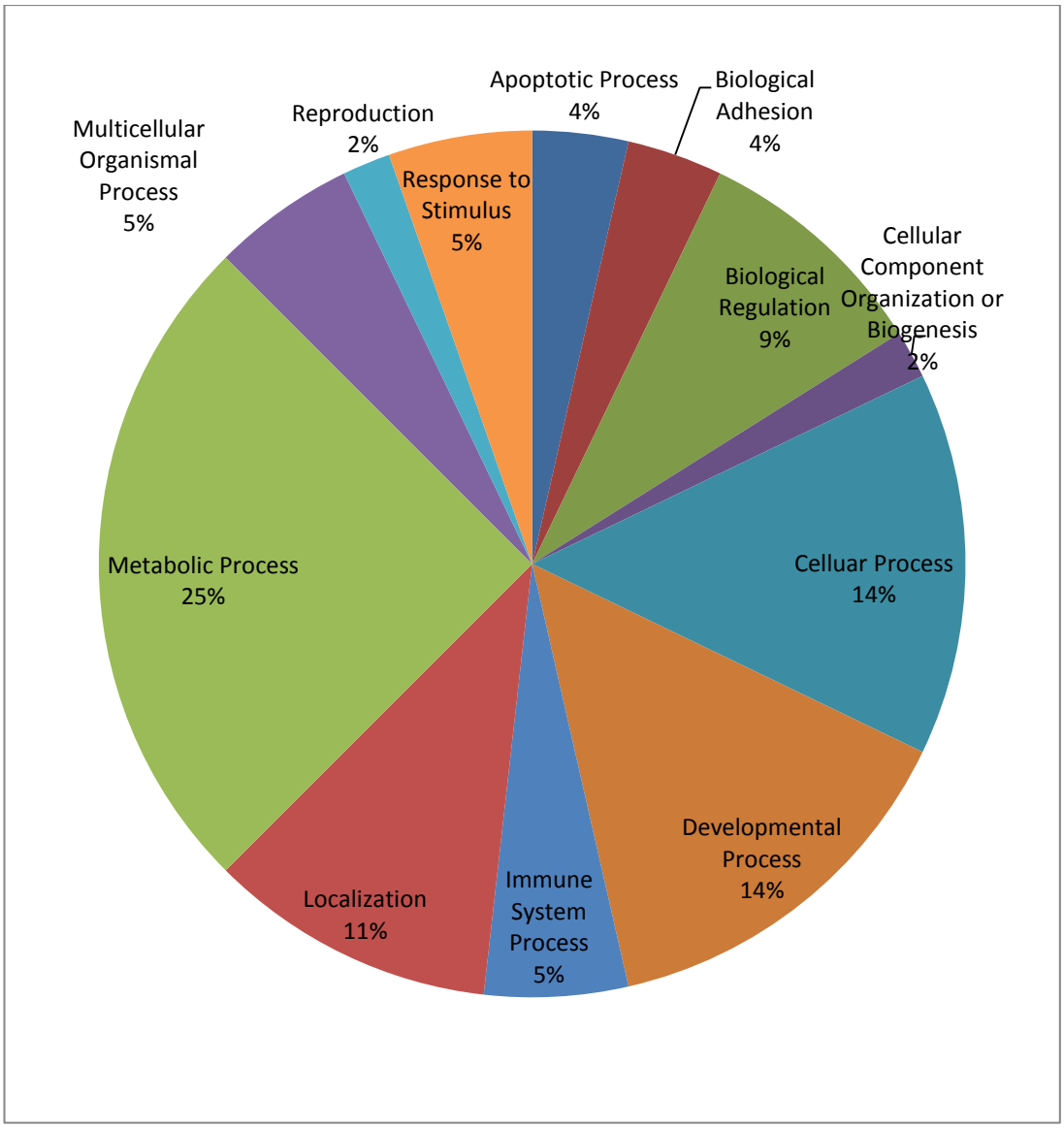

Figure 4.3: Pie chart results showing the biological processes associated with genes identified as differentially methylated between DHA and control group at 5 years of age as determined using the PANTHER Classification System.

4.4.1.2.1. DMRs between DHA and Control Group in Males and Females

When the number of DMRs between DHA and control group at birth and at 5 years of age were analysed separately in males and females, more DMRs were identified between the DHA group and control group in males compared to females at both time points (Table 4.4).

Multiple comparisons were undertaken to compare the DMRs between treatment groups in the different sexes at the different time points (Table 4.5). Interestingly, three of the DMRs between the DHA and control group in males (chr15:93652578-93653310, *KIF19* and *SLC6A18*) were identified both at birth and at 5 years of age (Table 4.5). The differences in methylation between treatment groups for these 3 DMRs were small, but consistent over multiple probes (Figure 4.4-4.6).

Table 4.4 Number of DMRs between the DHA and control group in separate analysis of males and females

		Number of DMRs
Birth	Male	150
	Female	79
5 years	Male	230
	Female	43

Table 4.5: DMRs between the DHA and control group that were found in multiple comparisons

hg19 coordinates	no. probes	gene symbol	gene name	group	DMR in comparison
chr1:175122830-175123194	3				birth females, 5 years males
chr10:103530053-103530255	4	FGF8	fibroblast growth factor 8	3'UTR,Body	birth females, 5 years males
chr10:88022641-88023243	7	GRID1	glutamate receptor, ionotropic, delta 1	Body	birth females, 5 years males
chr12:132293329-132293702	5				birth all, 5 years females
chr15:93652578-93653310	7				birth males, 5 years males
chr17:19628012-19628421	4				birth females, 5 years all, 5 years males
chr17:72350354-72350710	4	KIF19	kinesin family member 19	Body	birth males, 5 years males
chr3:138152837-138152902	2	ESYT3	extended synaptotagmin-like protein 2	TSS1500	birth females, 5 years males
chr3:138152837-138153439	11	ESYT3	extended synaptotagmin-like protein 3	TSS1500,TSS200,1st Exon,5'UTR	birth all, birth males
chr5:1245669-1246795	5	SLC6A18	solute carrier family 6 (neutral amino acid transporter), member 18	Body,3'UTR	birth males, 5 years males
chr6:150346721-150347053	10	RAET1L	retinoic acid early transcript 1L	TSS200,TSS1500	birth all, 5 years males
chr6:28945182-28945507	8				birth pools all, 5 years females

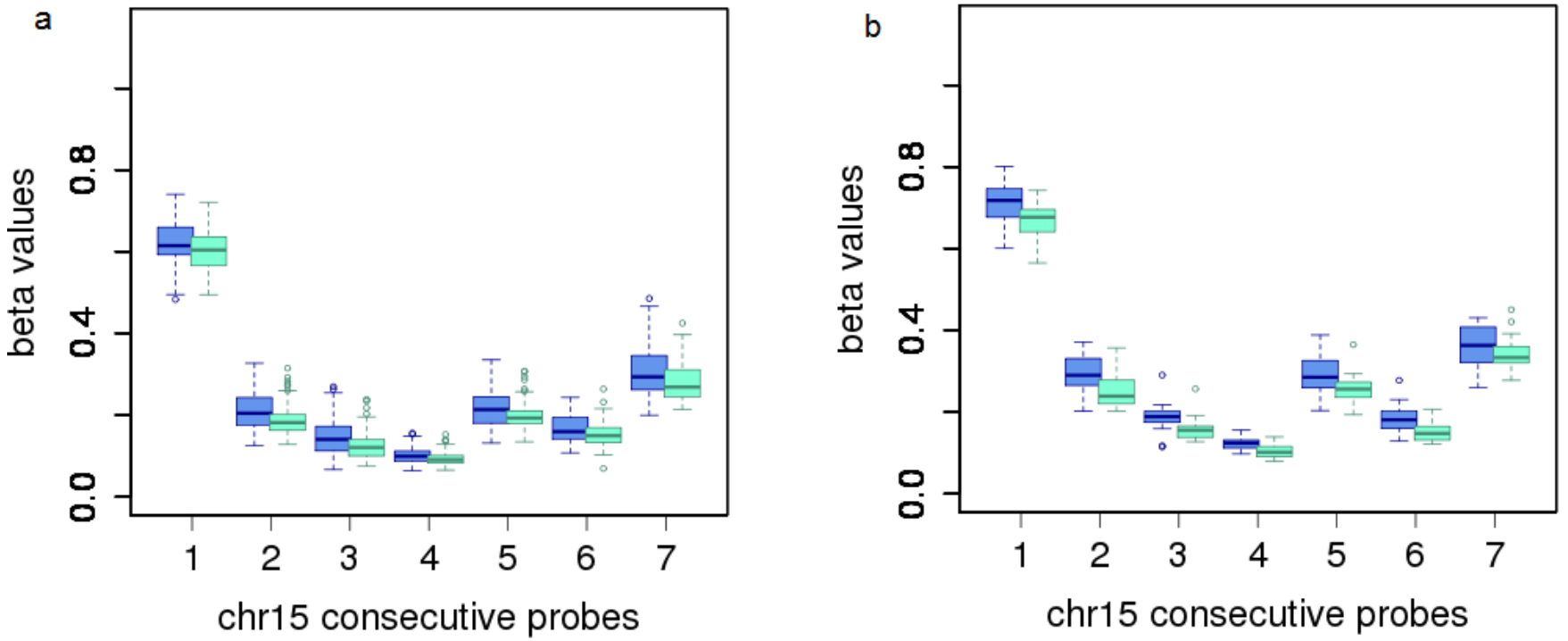

Figure 4.4: DMR multiple boxplots of chr15:93652578-93653310 between treatment groups (green box represents the control group and blue box represents the DHA group) in male a) at birth and b) at 5 years of age. The middle line in each box is the median methylation level in each group. The box shows values within the 25th and 75 percentile range and the upper/lower whiskers show a 95% confidence interval.

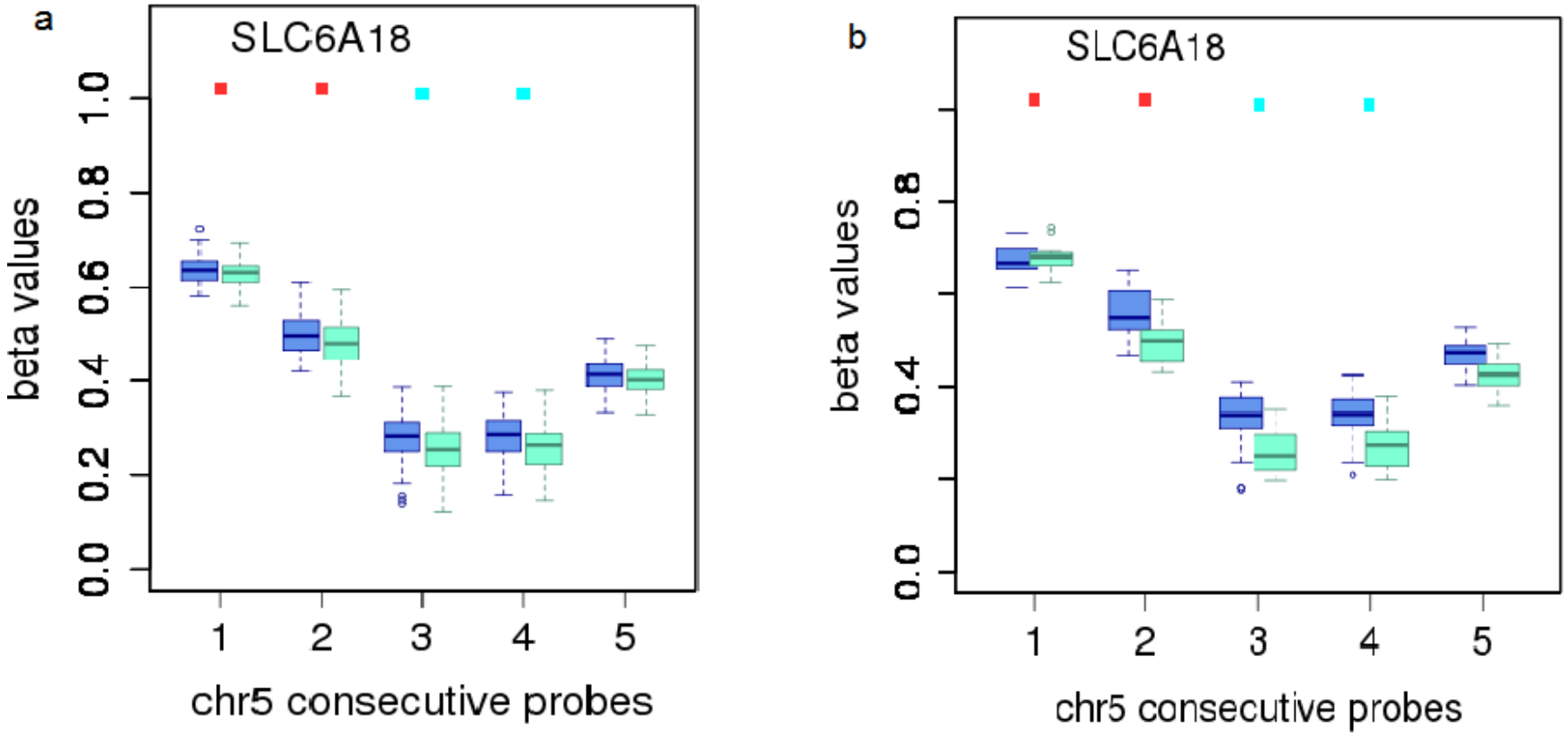

Figure 4.5: DMR multiple boxplots of SLC6A18 between treatment groups treatment groups green box represents the control group and blue box represents the DHA group) in male a) at birth and b) at 5 years of age. The middle line in each box is the median methylation level in each group. The box shows values within the 25th and 75 percentile range and the upper/lower whiskers show a 95% confidence interval.

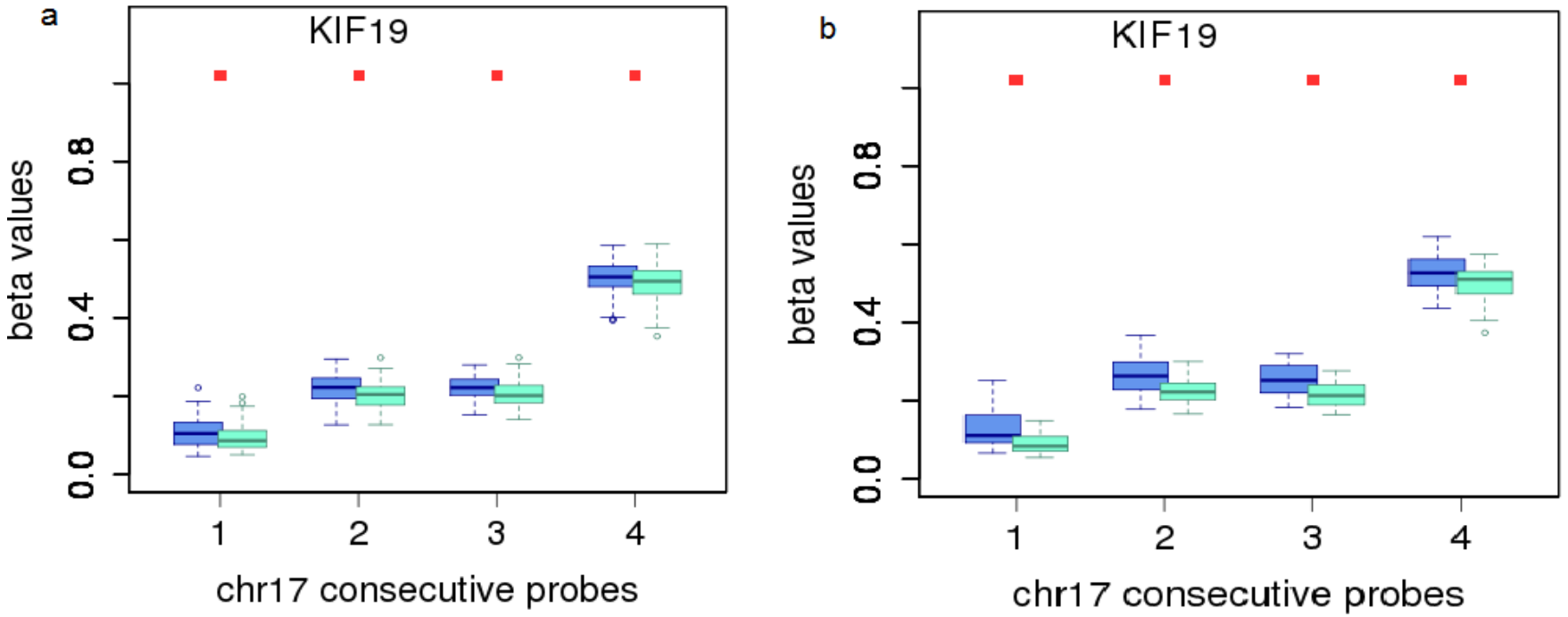

Figure 4.6: DMR multiple boxplots of KIF19 between treatment groups treatment groups green box represents the control group and blue box represents the DHA group) in male a) at birth and b) at 5 years of age. The middle line in each box is the median methylation level in each group. The box shows values within the 25th and 75 percentile range and the upper/lower whiskers show a 95% confidence interval.

4.5. Discussion

In the previous Chapter, it was reported that there was no effect of prenatal n-3 LCPUFA supplementation on global DNA methylation at either birth or 5 years of age. However, it was also demonstrated that prenatal DHA supplementation was associated with a significant relationship between global DNA methylation levels in blood at birth and percentage fat mass and plasma insulin and glucose levels in children at 5 years of age. This raised the possibility that exposure to an increased supply of n-3 LCPUFA before birth may alter DNA methylation in specific genes related to fat mass and/or insulin sensitivity/secretion. In the current Chapter, it was reported that while prenatal DHA supplementation was associated with small DNA methylation changes in specific genomic regions at birth and at age 5 years, there was little evidence of any effect of prenatal n-3 LCPUFA supplementation on DNA methylation patterns in individual genes or genomic regions related to fat deposition or insulin action. The results of this Chapter also suggested that males might be more susceptible to the effects of increased prenatal DHA exposure compared to females at an epigenetic level, indicating that it will be important to evaluate effects in males and females separately in future studies.

4.5.1. DMRs at Birth and at 5 Years of Age

DNA methylation analyses identified 44 DMRs at birth and 30 DMRs at 5 years of age between the DHA and control group in the combined population. Interestingly, the majority of these DMRs at both birth and 5 years of age showed higher methylation levels in the DHA group compared to the control group. Although the DMRs between treatment groups were different between birth and at 5 years of age, they were mainly clustered in metabolic, developmental and immune system processes at both time points. While it is important to note that no gene specific methylation changes were observed in this current study, the finding that maternal DHA supplementation had effects of methylation of genomic regions involved in immunological processes is interesting in the context of previous studies which have demonstrated that maternal n-3 LCPUFA supplementation during pregnancy can have immunologic effects. These studies have demonstrated effects of maternal n-3 LCPUFA supplementation on umbilical cord blood immune markers (blood cytokine mRNA[247], plasma cytokines[248], LTB4 production from neutrophils[249], cytokine production by mononuclear cells[250]) and an altered cord blood haematopoietic progenitor phenotype[251] which might be expected to impact on allergic sensitisation and on the development of atopic disease[252]. Olsen and colleagues have also previously reported that fish oil supplementation (2.7g n-3 LCPUFAs daily, n=266) in late pregnancy (from gestation week 30 until delivery) reduced the risk of asthma (p=0.03) and allergic asthma (p=0.01) in children at 16 years of age compared to olive oil treatment (n=136)[253]. This study suggested that increased intake of n-3 LCPUFAs during pregnancy can cause long-term effects on the immune function of the children[253]. Another large double blinded RCT (n=706) reported that maternal supplementation with n-3 LCPUFA (900mg daily) from 21 weeks' gestation until

birth decreased percentage of infants diagnosed as having atopic eczema (7% vs 12%, p=0.04) and sensitised to egg (9% vs 15%, p=0.02) compared to control group (vegetable oil)[254]. The results of the present study therefore suggest that these effects of prenatal DHA on subsequent immune function could potentially be mediated by epigenetic modifications.

Although clinical studies have reported that increased n-3 LCPUFA supplementation during pregnancy can improve immune system in children; there is very limited information to date as to the effects of n-3 LCPUFAs on the epigenetic status of gene/genome regions involved in immune regulation. Recently, a double blinded, randomised controlled trial reported that daily 400mg DHA supplementation from 18 to 22 week of gestation to parturition was associated with changes in DNA methylation levels in LINE-1 repetitive elements (p=0.03) in infants of mothers who smoked during pregnancy[32]. This same study also reported that DHA supplementation tended to result in altered methylation of the immune factors *TNF-α* and *IL13* in the cord blood (p=0.06) in the maternal smoking group[32]. Since the *IFNγ* and *IL13* genes encode Th1 and Th2 cytokines respectively, this result supports the suggestion that n-3 LCPUFA can act to alter the subsequent immune function of the offspring via epigenetic mechanisms. While there was no effect of maternal n-3 LCPUFA supplementation on the DNA methylation of *IFNγ* and IL13 in the current Chapter, this may be explained by the fact that the majority of mothers in our study were non-smokers.

While few DMRs were identified between DHA and control groups in the neonatal samples, one interesting finding was that the methylation of genetic regions encoding

the *CCK* (cholecystokinin) gene was increased in the DHA group compared to control group. *CCK* stimulates the release of digestive enzymes which catalyse the digestion of fat, protein, and carbohydrates, and therefore promote the hydrolysis of starch into sugars[255-261]. However, effect of changing methylation status of CCK on gene and protein levels need to be determined in future studies.

Of the DMRs identified at 5 years of age, *ARHFAP35* is of particular interest. *ARHFAP35* (Rho GTPase activating protein 35) has previously been shown to act as a switch between adipogenesis and myogenesis during differentiation of the common mesenchymal precursor that gives rise to both adipocytes and myocytes[262-264]. As a result, if the methylation differences observed in the Rho GTPase gene translated into differences in activity of this enzyme between the DHA and control groups, this could influence the fate of cells in the differentiation of adipocyte and myocyte precursors and alter the balance between muscle and fat cell formation[263]. However, since the results in Chapter 2 showed no difference in of the percentage fat mass in children in the DHA treatment compared to control group, the functional significance of the alteration in methylation of this gene is unclear.

4.5.2. Sex Differences in the Sensitivity to Increased Prenatal Exposure of N-3 LCPUFAs

The results of this Chapter indicated that there were more DMRs between the treatment groups in males compared to females at both birth and 5 years of age. Moreover, there were 3 DMRs (SLC6A18, chr15:93652578-93653310, and KIF19) in males which were identified at both birth and 5 years of age; but, no DMRs were found to be altered at both time points in females. *SLC6A18* is involved in kidney amino acid transport[265], and it has previously been reported that silencing of *SLC6A18* is associated with a defect in glycine uptake at the level of the late proximal tubule in mice[265,266]. The DMR of chr15:93652578-93653310 is intergenic and has not been associated with any known gene; so, the significance and potential physiological effects of this change are unclear. *KIF19* is a protein coding gene for kinesin family member 19[267] and there are limited studies on this gene. None of these three DMRs have previously been related to obesity and metabolic health; and further studies are required to examine this in more detail.

As discussed in Chapter 3, there are significant differences in metabolic systems and body composition between males and females. In addition, the data presented in Chapter 2 showed that males were more susceptible to negative effects of DHA supplementation on insulin sensitivity compared to females. The results are in line with data from numerous previous studies that have indicated that males are more susceptible to the programming effects of a large range of prenatal insults in comparison to females[79,208]. The results of the current Chapter add to this evidence, and suggest that males are more responsive to the effects of prenatal exposure to an increased supply of n-3 LCPUFAs compared to females on epigenetic level.

Although no previous studies have reported sex difference in the effect of increased prenatal n-3 LCPUFA exposure on the epigenome in offspring; it has been demonstrated in a number of previous studies that male offspring respond differently to other nutritional exposures *in utero* on an epigenetic level compared to female offspring[268]. For example, Castillo and colleagues reported in a rat study that methylation in CpG sites related to the phosphoenolpyruvate carboxykinase (*PEPCK*) and acyl-CoA oxidase (*AOX*) genes in the liver was altered in male offspring of dams that were given cadmium treatment (50 ppm) during pregnancy compared to the control group, but not affected in females[268]. Although the evidence is limited; these results raise the possibility that epigenetic response in offspring to the alteration of specific nutrients *in utero* might be sex dependent. This will be important to examine in more detail in future studies and highlights the importance of separating analyses by sex in studies of developmental programming.

4.5.3. Limitations

As discussed in Chapter 3, there are a number of limitations which need to be considered when interpreting the results of the epigenetic analyses presented in this thesis. One limitation is the fact that the DNA at birth and 5 years was isolated from different source material (dried blood spot at birth, buffy coat at 5 years of age). The results of this Chapter also suggested that there were not many genetic regions that were differentially methylated between treatment groups and that the difference of DNA methylation were small. This suggests that n-3 LCPUFA supplementation in the second half of pregnancy did not cause dramatic epigenetic changes in the infant/child. Furthermore, the relatively small changes induced by n-3 LCPUFA supplementation identified in the large sample of DOMInO children in which genome-wide methylation was assessed at birth (369 participants), could be difficult to replicate in the smaller number (65 participants) assessed at 5 years of age. This was most likely due to insufficient statistical power, which could at least partly explain why most of the DMRs detected at birth were not present at 5 years of age, although it may also be that the changes simply did not persist beyond the immediate neonatal period. Moreover, the n-3 LCPUFA supplementation in the DOMInO study was only given to women from about 20 gestational weeks until delivery, i.e. during mid to late pregnancy. This is potentially significant since previous studies have shown that nutritional exposures experienced during the peri-conceptional period and in early gestation have a more profound effect on the epigeneome and later metabolic health than the same nutritional exposure in late gestation[4,5,67,74,77,83]. This suggests that the timing of nutritional intervention is likely to be critical for its effect on the epigenetic status in offspring[9,11], and it is possible that the n-3 LCPUFA

supplementation in our study commenced too late in gestation to induce substantial epigenetic changes in the offspring.

4.5.4. Conclusion

To conclude, the results of this Chapter have shown that most of the alterations in DNA methylation in specific regions in children at birth and 5 years by supplementation of n-3 LCPUFAs during pregnancy were not related to glucose/insulin metabolism and fat deposition. The DMRs between n-3 LCPUFA and control groups at both birth and 5 years of age were, however, mainly clustered in metabolic, cellular, developmental and immune system processes and it is possible that the observed changes may therefore indirectly contribute to changes in the metabolic phenotype. The methylation level of two interesting regions, *CCK* and *ARHFAP35*, were increased in DHA group compared to control group, and these changes need to be validated in further studies. In addition, the results of this Chapter have suggested that the response to increased DHA exposure prenatally on an epigenetic level is more pronounced in males compared to females. While the effects observed were not large, small changes in DNA methylation in regulatory parts of the genome, such as in gene promotor or enhancers, may have biological relevance. However, whether this is the case in our study this needs further investigation. This study is the first to investigate the effect of increased prenatal exposure of n-3 LCPUFAs on DNA methylation later in life, and the results provide novel insights and potential future study directions regarding the mechanism of how maternal n-3 LCPUFAs supplementation during pregnancy affects children's health later in life. However, due to the limitations discussed previously, further studies are needed to confirm these results.

CHAPTER 5: OMEGA-3 FATTY ACID TREATMENT STIMULATES PROLIFERATION AND ALTERS GENE EXPRESSION IN THE FIRST TRIMESTER PLACENTAL TROPHOBLAST CELL LINE HTR8/SVNEO

5.1. Candidate Contribution

I was responsible all of the experiments including cell culture work and fatty acid analysis of the emulsions, cells and culture media. I also performed the proliferation assay and carried out all the RNA extraction from cells. I conducted all of the statistical analysis for the lipid composition and cell proliferation studies. I was also involved in discussions with bioinformaticians related to the analysis of the gene expression data and was responsible for the pathway analysis, tabulation and interpretation of the data.

5.2. Introduction

As discussed in the previous Chapters, evidence from *in vitro* and experimental animal and vlinical studies pointing to the beneficial effects of n-3 LCPUFA on fat deposition and insulin sensitivity have led to suggestion that prenatal exposure to increased DHA could improve metabolic health of the child. The results from Chapter 2 suggested however that maternal supplementation of n-3 LCPUFA from the second half of pregnancy was actually associated with reduced insulin sensitivity in children at 5 years of age. However, the underlying mechanism of how the nutritional environment *in utero* can impact on metabolic health later in life is unclear. While increasing evidence has suggested that epigenetic modifications play an important role in metabolic programming, the results from Chapter 3 and 4 of this thesis provide limited evidence to support a significant role of the epigenetic effects in mediating the effects of prenatal n-3 LCPUFA exposure on infant and child outcomes.

As the only organ connecting the mother and fetus, the placenta is the single most important organ for fetal development and ensures that essential nutrients are transferred to the fetus from the maternal circulation[46]. Moreover, the placenta also produces hormones involved in regulating fetal metabolism and growth[46,168]. Since the placenta has a crucial role in fetal development, changes or defects in placental structure and/or function have a significant impact on fetal development, and, consequently, the potential to influence long term health outcomes[46]. Previous studies have demonstrated that the placenta acts as an important mediator of the effect of a number of maternal nutritional perturbations (e.g. under-nutrition) during pregnancy on the development of the fetus, and therefore plays a critical role in

developmental programming[45]. Maternal factors affect the function of the placenta both in terms of nutrient transport and endocrinology and also placental structure, which alters both placental function and fetal vascular resistance[45,46,178]. A study which examined the association between under-nutrition during pregnancy and placental development in the Dutch Winter Hunger Famine cohort also reported that increased placental weight was associated with the combination of famine exposure in early pregnancy and high food intake in mid-later pregnancy[181]. Changes in placental function may cause redistribution of fetal blood flow and changes in the regulation of gene expression, which both have the potential to alter the normal pattern of development and, therefore, to have long-term effects on offspring health[46].

Early placental development, including the establishment of placental vascularisation, plays a critical role in subsequent placental function[190]. Recent studies have shown that n-3 LCPUFA treatment *in vitro* promoted angiogenesis during this period, which has the potential to enhance subsequent placental function by improving placental blood flow[190]. Both human and animal studies have also suggested that maternal DHA supplementation during pregnancy can increase the concentrations of anti-inflammatory resolvins, protectins[188] and specialised pro-resolving lipid mediators in the placenta[189]. While these previous studies suggest that n-3 LCPUFA during have effects on the placenta, there is still limited understanding of the specific impact of n-3 LCPUFA/DHA on placental structure and function and, therefore, whether the placenta could be a mediator of the programming effects of prenatal DHA exposure is unclear.

Therefore, the aim of this Chapter was to determine the impact of treatment with a DHA enriched fish oil emulsion on fatty acid composition, proliferation rate and gene expression profile in a human placenta first trimester cell line and to compare these effects with those resulting from treatment with a soy oil emulsion containing no n-3 LCPUFA.

5.3. Materials and Methods

5.3.1. Reagents

The human first trimester trophoblast cell line, HTR8/SVneo, was kindly provided by Professor Charles Graham (Queen's University, Kingston, Ontario)[171]. HTR8/SVneo cells used in this study were maintained in RPMI 1640 media (Sigma, St Louis MO, USA) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) and 2mM L-glutamine (Life Technologies, Grand Island NY, USA). Cells were incubated in a humidified atmosphere at 37ºC in 20% O_2 and 5% CO_2.

5.3.2. Fatty Acids Composition in Fish Oil (FO) and Soy Oil (SO) Emulsions

The fatty acid composition of the FO and SO emulsions is shown in Table 5.1. The FO emulsion contained predominantly DHA (81.3% of total fatty acids) and small quantities of other polyunsaturated and monounsaturated fatty acids. In contrast, the SO emulsion predominantly consisted of the n-6 PUFA linoleic acid (LA, 18:2n-6, 47%) and oleic acid (18:1n-9, 30.8%). Importantly, there was no overlap in the fatty acid compositions of the FO and SO emulsions. Both emulsions were kindly donated by Nu Mega Ingredients Pty Ltd (Altona North, VIC, Australia).

Table 5.1: Fatty acids composition of SO and FO

Fatty Acid	*SO Fatty Acids (%)*[1]	*FO Fatty Acids (%)*[2]
16:0	12.5	ND[3]
18:0	3.6	ND
18:1n-9	30.8	ND
18:1n-7	1.6	ND
18:2n-6	47.0	ND
18:3n-3	4.6	ND
20:5n-3	ND	5.6
22:5n-6	ND	6.3
24:1	ND	2.3
22:5n-3	ND	4.4
22:6n-3	ND	81.3

[1] Fatty acid (%) is present as % of total fatty acids in SO.
[2] Fatty acid (%) is present as % of total fatty acids in FO.
[3] ND: not detected.

5.3.3. Measurement of DHA Accumulation in HTR8/SVneo Cells

DHA accumulation was measured by plating $4x10^5$ HTR8/SVneo cells per well in a 6-well plate in RPMI 1640 media supplemented with 0.5% HI-FBS and 2nM L-glutamine (serum reduced media, SRM) at 37ºC, 20% O_2 and 5% CO_2 for 72 hours. The cells were then treated with 2mL SRM (no treatment), 2mL SRM containing 100μM FO emulsion or 2mL SRM containing 100μM SO emulsion for 16 hours. At 0, 3, 6, and 16 hours after treatment, cells and media from each treatment were collected for fatty acid analysis. Three replicate experiments were performed on 3 separate occasions for each treatment and time point.

The fatty acid composition of the cells and media was determined as previously described, following the same procedures as in Chapter 2[269]. Briefly, total lipids were extracted from cells and media with a chloroform/isopropanol (2:1, v/v) solvent system. The total lipids were methylated in 1% H_2SO_4 in methanol at 70°C for 3 h. After cooling, the resulting fatty acid methyl esters (FAME) were extracted into n-heptane and transferred into vials containing anhydrous Na_2SO_4 as the dehydrating agent. Butylated hydroxyanisole (0.005%, w/v) was added to all organic solvents except n-heptane to prevent fatty acid oxidation. FAMEs were separated and quantified using a Hewlett-Packard 6890 gas chromatograph (Hewlett Packard, CA, USA) equipped with a 50m capillary column (0.32mm ID) coated with BPX-70 (0.25μm film thickness, SGE Pty Ltd, Ringwood, Victoria Australia). The injector temperature was set at 250°C and the detector (flame ionization) temperature at 300°C. The initial oven temperature was 140°C and was programmed to rise to 220°C at 5°C/min. Helium was used as the carrier gas at a velocity of 35cm/s.

FAMEs were identified based on the retention time in comparison to that of authentic lipid standards obtained from Nu-Chek Prep, Inc. (Elysian, MN USA) .

5.3.4. Cell Proliferation

To assess cell proliferation, $2x10^4$ HTR8/SVneo cells per well were plated in a 96-well plate in SRM and incubated for 2 hours at 37ºC, 20% oxygen and 5% CO_2. Cells were then treated with 200µL SRM, or 200µL SRM containing 50µM or 100µM FO, or 200µL SRM containing 50µM or 100µM SO. The plates were incubated for 16, 24 or 48 hours at 37ºC, 20% O_2 and 5% CO_2. Cell viability was determined using the Calcein AM cell viability assay (Trengen, Gaithersburg, USA) according to the manufacturer's instructions. Six replicates from each treatment group and time point were tested in 3 independent experiments. The proliferation rate was calculated by dividing the average number of cells present in each treatment group by the average number of cells in the no treatment group at the same time point, and results expressed as a percentage (%) of the proliferation in the no treatment group.

5.3.5. Gene Expression Analysis

200,000 HTR8/SVneo cells per well were seeded in SRM in a 6-well plate and incubated for 24 hours at 37ºC in 20% O_2 and 5% CO_2. After 24 hours, 2mL of either SRM, SRM containing 50μM FO, or SRM containing 50μM SO were added. Each treatment consisted of 4 replicates. After 24 hours, 1mL TRIzol Reagent (Ambion, Victoria, Australia) was added to the wells. Total RNA was isolated from cells using TRIzol Reagent and purified using the Qiagen RNeasy Mini Kit (Hilden, Germany) according to the protocol provided by the manufacturer. The quality and concentration of the RNA was determined by measuring the absorbance at 260 and 280nm (NanoDrop Technologies, Wilmington, DE) and RNA integrity was quantified using an Agilent Bioanalyser to ensure all samples had RQI (RNA Quality Indicator) >7. RNA samples were hybridized to Illumina Human HT-12 v4 microarrays at the Adelaide Microarray Centre, Australia.

5.3.6. Microarray Data Analysis

The Illumina HT-12 v4 bead array data were pre-processed, quantile normalized and $\log_2$ transformed using the *beadarray* package[270]. Data quality was assessed by inspecting distance between arrays, array intensity distributions and MA-plots using the *ArrayQualityMetrics* package[271], with no outliers detected. All probes (47,318) were re-annotated using the *AnnotationDBI* package (IlluminaHumanv4.db), which includes mapping-based probe quality information[272]. Based on these annotations, data for probes with “no match” or classed as "bad" were removed from the dataset. Testing for differential expression between groups was performed using linear models and Empirical Bayes methods[273], with multiple testing corrected by calculating Benjamini and Hochberg FDR adjusted p-values as implemented in the *Limma* package[274]. Genes with FDR corrected p-values <0.01 and a fold-change <0.75 or >1.5 were considered to be differentially expressed between groups. All data processing and analyses were performed in the R environment, version 3.0.1.

5.3.7. Statistical Analysis of Lipid Composition and Cell Proliferation

Differences in the fatty acid composition of the cells and media following FO or SO treatment were assessed using a Student's unpaired *t*-test. The effect of FO and SO treatment on cell proliferation was assessed using a one-way analysis of variance (ANOVA). Where the one-way ANOVA revealed a significant effect, the Duncan's multiple range test was used post-hoc to identify significant differences between groups. $P<0.05$ was considered significant in all analyses. IBM SPSS Statistics 19 version for Windows (SPSS Inc., USA) was used for the statistical analyses.

5.4. Results

5.4.1. Effect of FO And SO Treatment on the Fatty Acid Composition of HTR8/Svneo Cells

After 16 hours, cells treated with fish oil (FO) exhibited a significant increase in DHA content (from 0.96% to 24.57% of total fatty acids) and total n-3 content (from 1.63 to 27.61% of total fatty acids) (Table 5.2). Accordingly, the levels of DHA and n-3 LCPUFA in the media as a percentage of total fatty acids decreased significantly across the same time period (Table 5.2). In the SO treated cells, LA content increased from 2.61% to 25.06% of total fatty acids and total n-6 PUFA content increased from 6.57% to 31.58% of total fatty acids following 16 hours of incubation, while the levels of the respective fatty acids in the media significantly decreased over the same time period (LA from 38.72% to 36.45% and total n-6 PUFAs from 39.12% to 36.93% of total fatty acids) (Table 5.2). There was also a small but significant decrease in the DHA content of SO treated cells (Table 5.2). There was no significant change in the fatty acid composition of either the cells or the media after the 16 hour incubation period in the no treatment group (Table 5.2).

Table 5.2: Percentage of different fatty acids in medium and HTR8/SVneo at 0 and 16 hours after treatment

	Medium						***Cells***					
	No Treatment		**SO**		**FO**		**No Treatment**		**SO**		**FO**	
	0 h	16 h	0 h	16 h	0 h	16 h	0 h	16 h	0 h	16 h	0 h	16 h
Total Saturates	98.94	98.87	24.52	24.28	54.73	58.82*	55.17	54.18	49.91	26.64*	55.17	42.96*
Total Trans	0.01	0.01	1.07	1.05	ND	ND	1.18	1.21	3.24	2.01	1.18	0.53
Total Monos	0.81	0.92	27.19	27.58	3.54	3.61	38.04	39.26	34.04	29.22*	38.04	23.58*
Total Omega 9	0.62	0.65	24.74	24.71	1.64	1.78	28.24	29.25	22.16	22.75	28.24	16.74
Total Omega 3	0.09	0.09	0.05	0.04	37.21	32.54*	1.63	1.34	2.67	4.34*	1.63	27.61*
20:5n-3	ND	ND	ND	ND	2.12	1.93*	0.81	ND	ND	0.47*	0.21	0.99*
22:5n-3	ND	ND	0.03	0.03	1.83	1.55*	0.41	0.42	0.77	0.91	0.41	2.06*
22:6n-3	0.09	ND	0.18	0.18	33.13	28.97*	0.96	0.92	1.91	0.75*	0.96	24.57*
Total Omega 6	0.16	0.15	39.12	36.93*	4.53	4.66	2.44	2.37	6.57	31.58*	2.44	4.61*
20:4n-6	0.16	0.15	0.24	0.36	0.66	0.63	1.31	1.22	2.31	2.53	1.31	1.07*
18:2n-6	ND	ND	38.72	36.45*	0.38	0.33	0.76	0.55	2.61	25.06*	0.76	0.77

∗: Values at 16 hours were significantly different compared with values at 0 hours (p <0.05) as determined by student t-test. Data are mean of n=3 per group.

ND: Not detected

5.4.2. Effect of FO and SO on Cell Proliferation

FO treatment at either 50 or 100μM significantly (p<0.05) increased the proliferation rate compared with no treatment at all time-points (Figure 5.1). The 50μM SO treatment also significantly increased the proliferation at 16 and 48 hours; but not at 24 hours (Figure 5.1a). The proliferation rate of cells treated with 50μM FO was significantly greater than those cells treated with 50μM SO at 16 and 48 hours. Cells treated with 100μM FO had a significantly higher proliferation rate than those treated with 100μM SO at 16 and 24 hours after treatment (Figure 5.1b).

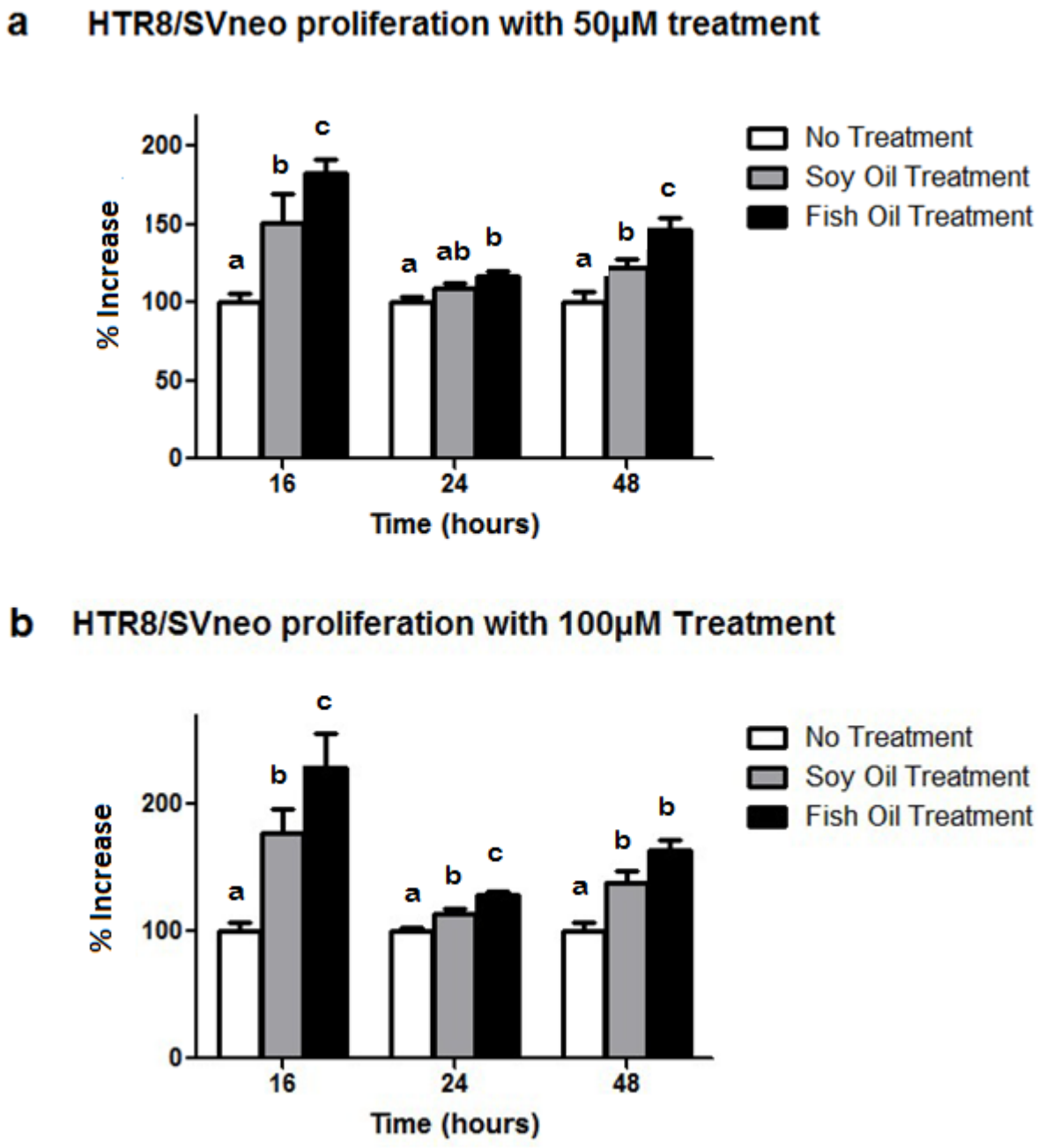

Figure 5.1: Effect of SO and FO treatment at 50µM (a) and 100µM (b) on cell proliferation following 16, 24 and 48 hours. Cell proliferation was measured using the Calcein-AM assay. Proliferation is expressed as a % increase which it is the % of increase compared with no treatment. Data represents mean ±SEM of 18 replicates. Values with different superscripts are significantly different from each other as determined by one-way ANOVA with Duncan post hoc multiple comparison test, *p*-value<0.05.

5.4.3. Genes Differentially Expressed between FO and No Treatment Groups

There were 139 array probes showing significant ($p<0.01$) differential expression between the FO and no treatment group (67 down-regulated, 72 up-regulated) (Figure 5.2). Pathway and network analyses generated by Ingenuity Pathways Analysis (IPA) software indicated that the 139 genes were clustered in development and function of the haematological system, tissue morphology and cell viability, cell signalling and development, lipid metabolism, cardiovascular and dermatological disease and related conditions (Table 5.3). The most significantly up-regulated genes were *GAR1* (Fold Change, FC: 3.88, p=0.0029), *MAGEB3* (FC: 3.86, p=0.0081) and *CAST* (FC: 3.21, p=0.004) (Table 5.4). *GAR1* is a member of the small nucleolar ribonucleoproteins gene family involved in rRNA processing and modification[275]. *MAGEB3* (melanoma antigen family B3) belongs to a gene family coding for proteins which direct the expression of tumor antigens associated with human melanomas[276]. *CAST* (calpastatin) is an endogenous calpain (calcium-dependent cysteine protease) inhibitor which is involved in muscle protein degradation[277]. None of these three genes have previously been reported to be associated with preterm delivery, placental function or in relation to n-3 LCPUFA. The top 3 down-regulated genes were *ANGPTL4* (FC: 0.05, p=0.00000125), *MGLL* (FC: 0.23, p=0.0094) and *SAMD4A* (FC: 0.37, p=0.0042). *ANGPTL4* (angiopoietin-like 4) encodes a protein which functions as a serum hormone that regulates glucose homeostasis, lipid metabolism, and insulin sensitivity[278-280], and also plays a role in cellular growth and proliferation, tissue development and placental angiogenesis[190,279]. *MGLL* (monoglyceride lipase) encodes a serine hydrolase that catalyses the conversion of monacylglycerides to free fatty acids and glycerol[281], while *SAMD4A* (sterile alpha

motif domain containing 4A) is a post-transcriptional regulator which binds to a RNA sequence motif known as the Smaug recognition element[282].

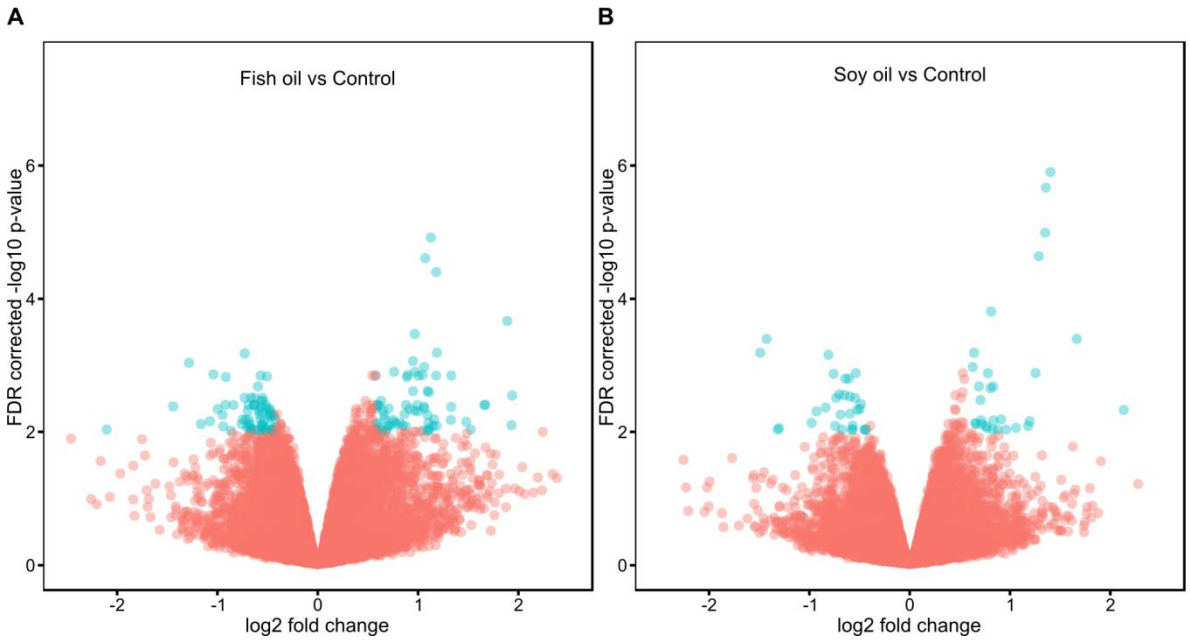

Figure 5.2: Volcano plots representing gene expression differences in HTR8/SVneo cells treated with FO emulsion (A) and SO emulsion (B) compared to no treatment. Aqua coloured dots represent array probes showing a significant difference (FDR corrected p-values<0.01) between FO/SO treatment and no treatment (expressed as control in the figure).

Table 5.3: Function networks modulated in HTR8 cells by SO or FO treatment as assessed by IPA

	No treatment vs SO		*No treatment vs FO*	
	Networks	**Score**	**Networks**	**Score**
1	Lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism	45	Haematological system development and function, tissue morphology, cell death and survival	54
2	Cell-to-cell signalling and interaction, cellular growth and proliferation, endocrine system development and function	36	Cell signalling, vitamin and mineral metabolism, cellular development	43
3	Embryonic development, organ development, organismal development	15	Cardiovascular disease, congenital heart anomaly, developmental disorder	29
4	Cellular development, cellular growth and proliferation, cell-to-cell signalling and interaction	2	Dermatological diseases and conditions, development disorder, hereditary disorder	26
5			Lipid metabolism, small molecule biochemistry, cell-to-cell signalling and interaction	24

Table 5.4: Top 10 up- and down- regulated genes in SO and FO groups compared with no treatment groups

	SO						***FO***					
	Up			**Down**			**Up**			**Down**		
	Gene	**FC**	**Adjusted *p***	**Gene**	**FC**	**Adjusted *p***	**Gene**	**FC**	**Adjusted *p***	**Gene**	**FC**	**Adjusted *p***
1	STON1	4.422374	0.00482	ANGPTL4	0.013062	2.49E-08	GAR1	3.876	0.002874	ANGPTL4	0.050	0.00000125
2	SREBF1	2.656846	0.00000126	F3	0.369185	0.00041	MAGEB3	3.855	0.008081	MGLL	0.230	0.009387
3	ASNS	2.559882	0.0000103	TOP	0.398636	0.009518	CAST	3.209	0.003972	SAMD4A	0.37	0.004214
4	MYLIP	2.44855	0.0000233	SAMD4A	0.402265	0.009048	TMPO	2.903	0.009402	F3	0.408	0.000924
5	ASNS	2.394055	0.00134	MARCH4	0.504606	0.00757	DSG2	2.813	0.00718	SH2D5	0.443	0.007772
6	CDH24	2.29877	0.007126	CDH5	0.52209	0.005053	COL4A3BP	2.533	0.004276	DDAH1	0.472	0.007026
7	FADS2	2.274653	0.008399	KCNH1	0.556398	0.004444	ZNF641	2.527	0.006734	KCNH1	0.483	0.001387
8	SALL4	1.95072	0.009518	PLIN2	0.587875	0.001379	DHRS3	2.292	0.00065	SERPINE1	0.498	0.004606
9	KIAA0367	1.88457	0.006697	MCL1	0.598611	0.003178	FBXW7	2.288	0.008223	MARCH4	0.515	0.005637
10	MFAP4	1.838203	0.009518	JUN	0.611565	0.00282	ASNS	2.282	3.95E-05	FOSL1	0.517	0.0084

5.4.4. Genes Differentially Expressed between SO and No Treatment Groups

There were 60 array probes (31 down-regulated, 29 up-regulated) which were differentially expressed between the SO and no treatment groups (Figure 5.2). The majority of these 60 probes corresponded to genes involved in lipid, vitamin and mineral metabolism, cell signalling, cell growth and endocrine development and embryonic/organ development as determined by Ingenuity Pathway Analysis (Table 5.3). The top 3 up-regulated genes in SO were: *STON1* (FC: 4.42, p=0.0048), *SREBF1* (FC: 2.66, p=0.000001) and *ASNS* (FC: 2.56 p=0.00001). *STON1* (stonin1) is involved in intracellular protein transport and endocytosis[283]. *SREBF1* (sterol regulatory element binding transcription factor) encodes a transcription factor which regulates the expression of a number of genes involved in lipid biosynthesis [284,285] and *ASNS* (asparagine synthetase) is involved in the synthesis of the amino acid asparagine [286]. The top 3 down-regulated genes were *ANGPTL4* (FC: 0.013, $p=2.5\times10^{-8}$), *F3* (FC: 0.37, p=0.0004) and *TOP* (FC: 0.40, p=0.009518). *F3* codes for a coagulation factor which is a cell surface glycoprotein[287]. *ANGPTL4* has been shown to be involved in placental angiogenesis and *TOP* contributes to the regulation of placental trophoblast differentiation and placental fatty acid uptake[170].

5.4.5. Genes Differentially Expressed between FO and SO Groups

There were only four genes (*ANGPTL4*, *DHRS3*, *NFAT5* and *PPFIBP1*) which were differentially expressed between the FO and SO group, and all were expressed at a higher level in the FO compared to the SO group. As indicated above, *ANGPTL4* (FC: 3.84, p=0.0046) plays a role in placental angiogenesis. *DHRS3* (FC: 1.94, p=0.008), dehydrogenase/reductase member 3 catalyses the oxidation/reduction of a wide range of substrates including retinoids and steroids[288]. *NFAT5* (FC: 2.09, p=0.008), nuclear factor of activated T-cells 5, is vital in inducible gene transcription during the immune response[289], and previous studies have shown that the expression of this gene is significantly up-regulated in preeclamptic compared to normal placentas[290]. *PPFIBP1* (FC: 1.77, p=0.008) is involved in cell adhesion[291], but there are no previous reports of this gene's involvement in placental function.

5.4.6. Genes Differentially Expressed in both FO and SO Groups Compared to No Treatment

There were 37 differentially expressed genes common to both of FO and SO groups in comparison to the no treatment group (Figure 5.3). The IPA results indicate that these 37 genes are involved in three main networks: 1) Cellular growth and proliferation, connective tissue development and function and hematopoiesis; 2) Cardiovascular system development and function, organ morphology, cellular growth and proliferation; and, 3) Lipid metabolism and molecular transport. The most represented pathways were cellular growth and proliferation and lipid metabolism. The direction of the change in expression relative to the no treatment group was the same for the FO and SO groups for the majority of genes. However, 3 genes (*INSIG1*, *MYLIP* and *SREBF1*) were down-regulated in FO compared with no treatment but up-regulated in SO compared with no treatment.

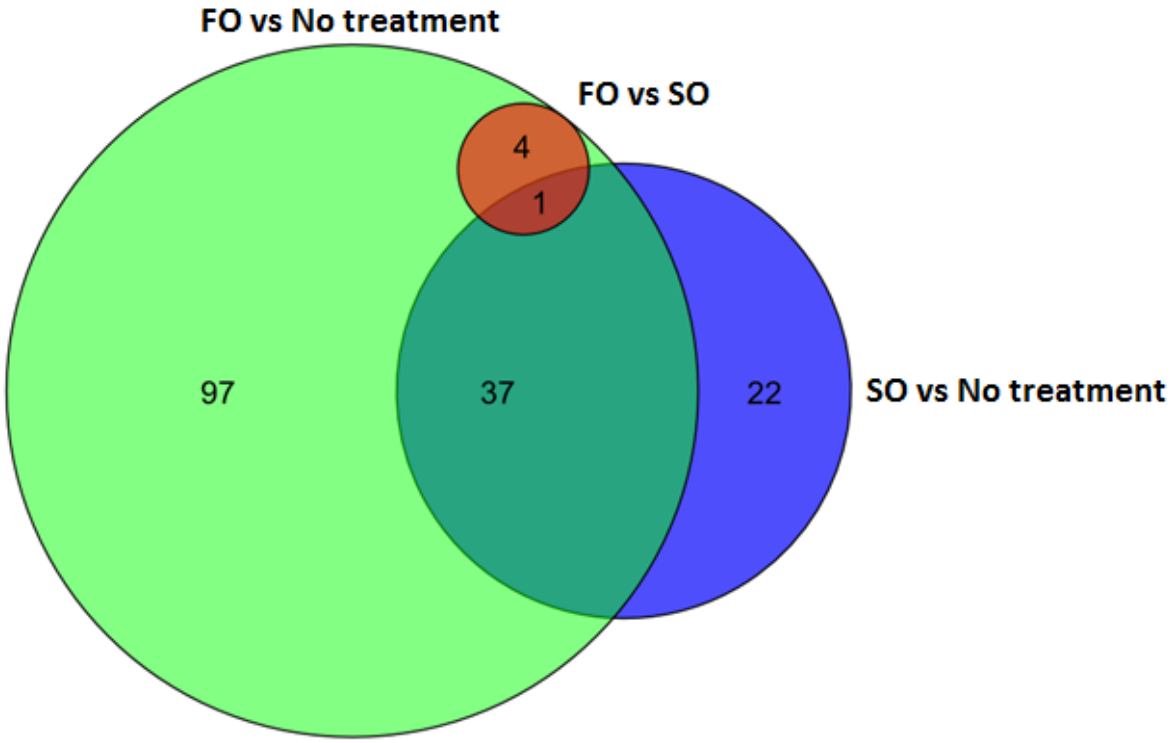

Figure 5.3: Euler diagram representing overlap of probes differentially expressed (FDR p-value <0.01) between the different treatment groups.

5.4.7. Genes Differentially Expressed in the FO Compared with No Treatment Group

There were 96 genes differentially expressed in the FO compared with no treatment group and unique to this treatment. According to the results from IPA, these 96 genes are clustered in pathways involved in 1) cardiovascular system development and function, cancer and gastrointestinal disease; 2) cancer, cell cycle, cell-to-cell signalling and interaction; 3) cellular development, nervous system development and function, connective tissue disorders; and, 4) neurological disease, ophthalmic disease, cardiovascular disease.

5.5. Discussion

The results of this study have demonstrated that fish-oil treatment increases the proliferation rate of a first trimester trophoblast cell line *in vitro* and is more effective in doing so than the same dose of SO. Both FO and SO treatment also induced significant alterations in gene expression of this trophoblast cell line after 24 hours of incubation in both overlapping and unique pathways, and I have identified novel candidate genes with a role in placental development that may be targets of DHA's effects on placental development *in vivo*.

5.5.1. FO and SO both Increase Proliferation of HTR8/Svneo Cells

The finding that the proliferation of *HTR8/SVneo* cells was significantly increased by both FO and SO treatment is consistent with previous studies in which *HTR8/SVneo* cells were treated with pure DHA or AA[190,292]. The analysis of gene expression identified 37 genes that were differentially expressed in both the FO and SO groups compared to controls. Importantly, the majority of these genes were involved in cellular growth and proliferation and were up-regulated in both the FO and SO groups. These common gene expression changes induced by both FO and SO treatment are consistent with the increased cell proliferation induced by both of these treatments. Since there appeared to be no overlap in the principal fatty acids present in the FO and SO treatments, these results suggest that an increase in the availability of fatty acids (or possibly energy), independent of the specific fatty acid involved, can affect placental cell proliferation at the gene expression level.

While both FO and SO promoted cell proliferation in *HTR8/SVneo* cells, FO had a more pronounced effect. This is consistent with previous studies in which n-3 LCPUFA increased HTR8/SVneo proliferation to a greater extent than a number of other fatty acids, including n-6 PUFA[190]. Thus, it appears that FO acts via additional pathways to those induced by SO, in promoting cell proliferation. In comparison to SO, FO treatment was associated with a significantly greater increase in the expression of 3 genes known to be involved in placental development, specifically *ANGTPL4*, *NFAT5* and *PPFIBP1*[288,289,291]. These genes have previously been implicated in the stimulation of placental angiogenesis and inhibition of cellular apoptosis. Hence, the greater capacity of FO to increase cell proliferation might be due to regulation of this unique set of genes compared with SO treatment.

5.5.2. The Effect of FO Treatment on Gene Expression in HTR8/Svneo Cells

The majority of genes influenced by FO treatment involved genes in pathways related to cardiovascular system function and development. This finding is consistent with the established role of n-3 LCPUFA in regulating processes important for cardiovascular function and disease progression, including platelet aggregation and angiogenesis in adults[15,111]. It is also in line with more recent studies demonstrating that n-3 LCPUFA supplementation during the perinatal period prevented the age-related increase in carotid intima-media thickness in individuals of low birth weight[293]. In addition, other studies have reported that maternal n-3 LCPUFA supplementation has favourable effects on cardiovascular function in their infants, in particular increasing heart rate variability[294,295]. Importantly, many of the processes that are involved in cardiovascular development and function are also important for early placental development, in particular angiogenesis. Contrary to our expectations, given the significant literature reporting anti-inflammatory actions of n-3 LCPUFA[111], FO treatment appeared to have limited effects on the expression of genes involved in immune and inflammatory pathways in this first trimester trophoblast cell line. It is possible, however, that FO treatment has different effects on the first-trimester placenta compared to later in gestation, and it will be important to address this in future studies.

Interestingly, the 4 genes that were differentially expressed between the FO and SO groups were involved in angiogenesis, apoptosis and placental function, and all of these genes were expressed more highly in the FO compared to SO group. *DHRS3* is known to promote local availability of retinoic acid[288], a factor which plays an important role in embryogenesis and maintenance of pregnancy[296-298]. It has been

shown that inflammation results in almost complete suppression of hepatic *DHRS3* expression in adult rats[299]. Together with the higher expression of the angiogenic factor, *ANGPTL4*, this suggests that FO treatment would have a more favourable effect on placental development, especially in relation to vascularisation, in comparison with SO. The expression of *PPFIBP1* has previously been reported to be suppressed by *VEGF* treatment during angiogenesis in human microvascular endothelial cells, human umbilical vein endothelial cells and telomerase-immortalised microvascular endothelial cells[300]. Thus, the finding of higher *PPFIBP1* expression following DHA treatment is somewhat at odds with the results of a previous study by Johnsen and colleagues, in which *VEGF* expression was increased by DHA treatment of HTR8/SVneo cells[190]. Further studies are required to fully characterise the role of *PPFIBP1* in placental function.

The expression of *NFAT5*, a protein involved in immune function, myogenesis and cancer invasion, was also higher in the FO compared to the SO group. Although no previous studies have explored the role of *NFAT5* in the proliferation of trophoblast cells, it has been reported that homozygous *NFAT5* null mouse embryos fail to develop normally and die after 14.5 days of embryonic development due to abnormal cardiac differentiation of stem cells[301]. In addition, other members of the *NFAT* family have been reported to be involved in promoting carcinoma invasion [302]. While this raises the possibility that *NFAT5* may play a role in the regulation of cell proliferation and invasion, more studies are needed to fully characterise the role of *NFAT5* in trophoblast cells in which these two processes feature.

5.5.3. SO *vs* No Treatment

The genes that were uniquely differentially regulated by SO compared to the no treatment group were involved in lipid, vitamin and mineral metabolism; pathways which were not identified in the FO vs no treatment comparison. This suggests that SO, i.e. n-6 PUFA, treatment, activates a separate set of pathways to those induced by FO treatment. In particular, the individual genes that were most affected by the SO treatment included genes involved in intracellular protein transport, amino acid and lipid biosynthesis. These results are consistent with previous data suggesting that n-6 PUFA promote lipid deposition and expression of lipogenic genes, including *SREBP1*[284,285], but to the best of my knowledge no previous studies have reported the effect of n-6 PUFA on the other genes identified in this study.

5.5.4. Limitations

The cell line used in the this experiment was derived from human first trimester trophoblast cells and it is possible that the effects of fatty acids on this cell line differ from those on placental cells *in vivo*, or later in gestation. Therefore, it will be important in future studies to determine whether n-3 LCPUFA/n-6 PUFA have different effects on more mature placental cells. In addition, conducting studies in placental explants derived from human placentas at different stages of gestation will be important to confirm that the results of the present study can be replicated in primary cell cultures.

5.5.5. Conclusion

This Chapter has clearly demonstrated that FO has an effect on both the growth rate and gene expression profile of human first trimester trophoblast cells *in vitro* which are consistent with enhanced proliferation and vascularisation of placental cells in early pregnancy, both of which could contribute to improved placental function later in gestation. The results from this Chapter have also have shown that both FO and SO treatment alters expression of a large number of genes which have not previously been shown to be related to placental function and/or fatty acids. While further studies are clearly required, this study provides evidence supporting the ability of n-3 LCPUFA to modulate placental structure and function, and suggest that the effects of DHA on fetal developmental could be mediated, at least in part, by effects on the placenta.

CHAPTER 6: GENERAL DISCUSSION

The current epidemic of obesity and type 2 diabetes is a major health issue world-wide; and the incidence of these diseases continues to increase[1]. Treating obesity and type 2 diabetes is difficult and costly, and once the conditions develop they are difficult to reverse[8,50,52,113]. Therefore, there has been growing interest in identifying strategies that can be applied early in life which can lower the risk of an individual developing these diseases in order to reduce the population burden of poor metabolic health. Epidemiological, clinical and experimental animal studies suggest that besides genetic factors, environmental exposures early in life are important determinants of the susceptibility of individuals to a range of diseases, including obesity and type 2 diabetes, later in life[2,8,50]. The effects of these early life exposures persist through the life course, and this phenomenon is therefore termed the "developmental origins of health and disease"[4,5,72,74,77,79]. The nutritional environment an individual experiences before birth and in early infancy is particularly vital for appropriate development and health through the life course, and exposure to an inappropriate nutrient supply is associated with an increased susceptibility to disease later in life[4,5,72,74,77,79]. While the mechanisms underlying this are not completely understood, an increasing number of studies have implicated modifications to the epigenome as playing a central role[215,303]. However, most of this evidence has come from animal studies, and there was limited direct evidence for an effect of nutritional exposures *in utero* on the epigenome of human infants and children.

In addition to global over/under-nutrition, there is growing evidence that exposure to altered levels of specific macro or micronutrients can also have programming

effect[215,303]. A number of recent studies have focussed on the effects of prenatal exposure to an increased supply of the n-3 LCPUFAs, DHA and EPA, on the fetus and child. These fatty acids, in particular DHA, are known to play a critical role in the development of the brain and nervous system[14]. More recently, however, there has been increasing interest in their metabolic effects, with some studies suggesting that maternal DHA supplementation may reduce the risk of metabolic conditions such as obesity and type 2 diabetes in the child[16]. However, there was a lack of consistent evidence from human studies to confirm if increasing the supply of n-3 LCPUFAs during early development could improve the metabolic health of the neonate after birth. Furthermore, whilst there was some prior evidence to suggest that maternal DHA supplementation could alter methylation in a small number of candidate genes, no previous studies had investigated the impact of prenatal DHA exposure on methylation status across the genome in human neonates or children. This thesis therefore aimed to investigate the effect of maternal DHA supplementation during pregnancy on insulin sensitivity in the child. Furthermore, I also aimed to determine the effects of increased prenatal DHA exposure on global methylation and methylation level in specific regions in blood sample collected from the children at birth and later in childhood. In addition to the epigenetic effects of prenatal n-3 LCPUFA supplementation in the children, the effects of increased exposure to n-3 LCPUFA on the placenta were also investigated *in vitro*, because those studies in animal models have suggested that much of developmental programming is likely to be mediated by effects on the placenta[38,39].

The first experimental Chapter of this thesis aimed to determine the effect of maternal DHA supplementation on insulin sensitivity and body composition of the

child at 5 years of age. The results of this Chapter indicated that maternal supplementation with high-dose DHA from 20 gestational weeks until delivery did not alter body composition, but was associated with decreased insulin sensitivity in the children. This was different to our hypothesis, based on previous evidence from studies in animal models and human adults that increased prenatal DHA would improve insulin sensitivity in the child. This suggests that prenatal exposure to increased n-3 LCPUFA has very different effects on the developing fetus than it does in adult humans. It is also important to note that previous studies have not produced consistent results about the effects of n-3 LCPUFA supplementation on glucose tolerance and insulin sensitivity, and have been limited by small sample sizes and samples not representative of the general population.

The results from Chapter 2 provided the first evidence that increased exposure to DHA during development could have long-term effect on insulin sensitivity in childhood from a large and robustly-designed randomised controlled trial. The results obtained in Chapter 2 also suggested that maternal n-3 LCPUFA supplementation during pregnancy had sex-specific effects on insulin sensitivity in children; when the data from children at 5 years of age were analysed separately by sex, HOMA-IR, fasting plasma glucose and insulin level were higher in DHA group compared to control group only in males but not females. This supports previous evidence which has shown that males are more susceptible to the effect of a range of early life nutritional exposures on insulin and glucose homeostasis[79,208]. Although the mechanism underlying the greater susceptibility of males to the effects of prenatal exposure on subsequent insulin sensitivity is unclear, it may be due in part to sex differences in body composition, glucose and insulin action, energy balance and

hormones environment, which are well described. Males and females have different adipose tissue distribution, and males typically have more visceral and hepatic adipose tissue that is associated with increased insulin resistance[209,210]. Moreover, the level of insulin-sensitising hormone, adiponectin, is higher in females compared to males which further contributes to an increased risk of insulin resistance/ type 2 diabetes in males compared to females. These differences might therefore contribute to the increased susceptibility to negative effects of prenatal DHA exposure on insulin sensitivity in males compared to females[209]. Overall, the results of this Chapter suggested that increased prenatal DHA has sex-specific effects on insulin sensitivity in the child and could potentially increase the susceptibility to developing type 2 diabetes in males.

While the results from Chapter 2 suggested that increased exposure of n-3 LCPUFAs during fetal development could have long-term effects on insulin sensitivity in children at childhood, it did not provide any insight into the mechanisms underlying this effect. While emerging evidence had suggested that epigenetic modifications, especially altered methylation, plays an important role in nutritional programming of metabolic diseases, few studies had been conducted in humans. Similarly, information as to the effect of maternal n-3 LCPUFA supplementation during pregnancy on global and genome-wide specific methylation in the child was also limited. In Chapter 3 of this thesis, I found no effect of maternal n-3 LCPUFA supplementation on global methylation level of the children either at birth or at 5 years of age, either in the population overall or when data from males and females were analysed separately. This result is consistent with results of a previous double blinded randomised controlled trial which showed that 400mg daily supplementation

DHA from 18-22 gestational weeks until birth didn't change the global DNA methylation level in the cord blood[32]. Together, the results from both Chapter 3 and this previous study suggest that maternal DHA supplementation during the second half of pregnancy has no significant effect on children's global methylation level either at birth or later in childhood.

While I did not find any effects of maternal n-3 LCPUFA supplementation on global methylation levels in the children both at birth and at 5 years of age, the relationships between global methylation and both percentage body fat mass percent and plasma glucose levels in the children at 5 years appeared to be altered by prenatal n-3 LCPUFA supplementation. This raised the possibility that maternal supplementation of n-3 LCPUFA from the second half of pregnancy might potentially affect DNA methylation in some specific genes/regions that are linked to fat deposition and insulin sensitivity, and that these changes persisted until 5 years of age. Hence, Chapter 4 investigated changes in DNA methylation in specific genes or regions caused by maternal n-3 LCPUFA supplementation during the second half of pregnancy using a genome-wide methylation approach. While prenatal DHA supplementation was associated with small DNA methylation changes in specific genomic regions at birth and at 5 years of age, most of the genes and genomic regions which were identified as being differentially methylated were not reported previously to be related with fat deposition or insulin action. This result might suggest that increased prenatal exposure of n-3 LCPUFAs didn't alter DNA methylation level in genomic regions related to insulin and glucose homeostasis and fat deposition. However, biological pathways analysis revealed that most of DMRs between DHA treatment group and control group were clustered in metabolic,

cellular, developmental and immune system processes, suggesting that DHA treatment during fetal development might alter DNA methylation to induce changes in these biological processes and might thereby indirectly influence insulin action.

While there was no DMRs which were identified at both birth and 5 years of age in the overall population; there were three interesting DMRs (*SLC6A18, chr15:93652578-93653310, and KIF19*) identified at both these time-points in males Together with the results of Chapter 2, indicating that males (but not females) in the DHA group had worse insulin sensitivity and higher fasting glucose levels than controls this raises an interesting question as to whether these three DMRs (*SLC6A18, chr15:93652578-93653310, and KIF19*) have a role in mediating the effects of prenatal DHA on insulin sensitivity after birth. However, no study has been done to investigate the association between DNA methylation level of these regions and metabolic health, and thus further studies are needed to answer the question. Moreover, there were more DMRs between two different treatment groups in males compared to females at both time points, which also provides additional data to suggest that males are more susceptible to the effects of prenatal DHA supplementation at an epigenetic level. This result further emphasises the importance of separating males and females in studies of developmental programming.

Another interesting result presented in Chapter 3 of this thesis was that there was a higher level of global DNA methylation level in children at 5 years of age compared to at birth, independent of treatment group or sex, suggesting increasing age, at least in the first few years of life, is associated with an increase in the global DNA methylation levels in blood. Interestingly, this finding was in contrast to the results of

a number of previous studies, which have shown that aging is associated with a *decrease* in global DNA methylation[224,225]. However, these previous studies have been conducted in adults and the majority didn't assess global DNA methylation in same individuals at different ages. Our study is the first to provide evidence that the global DNA methylation level increases with age across the first few years of life in humans. These results are consistent with the only other study to track global methylation in early life which also showed increased methylation between birth and 18 months of age. These results imply that methylation levels may increase across early postnatal life, before presumably reaching a maximal value and then declining across later adulthood. The reason for this is currently unclear, but may reflect continued development of the epigenome across the first few years of life.

The placenta is one of the most important organs during fetal development and previous studies had suggested an important role of the placenta in mediating the effect of maternal environment on the fetus. Chapter 5 aimed to investigate how n-3 LCPUFA treatment affected placental growth and gene expression, using an *in vitro* model. The results showed that n-3 LCPUFA increased the n-3 LCPUFAs content and stimulated proliferation in human first trimester trophoblast cells *in vitro* suggesting that DHA could potentially enhance placental growth and development in early pregnancy, and thereby improve placental function later in gestation. Moreover, the gene expression profile of human first trimester trophoblast cells was also affected by n-3 LCPUFA treatment. The majority of genes influenced by n-3 LCPUFA treatment involved genes in pathways related to cardiovascular system function and development which is consistent with the established role of n-3 LCPUFA in regulating processes important for cardiovascular function[15,111]. The

results of Chapter 5 also indicated that n-3 LCPUFA treatment altered the expression of genes involved in inflammation (*DHRS3*, *NFAT5*) and angiogenesis (*ANGPTL4*) raising the possibility that n-3 LCPUFA could improve placental function by increasing vascularization and/or decreasing inflammatory pathways. The significant impact of n-3 LCPUFA treatment on the placenta in this Chapter supports the suggestion that the effects of DHA on the fetus could potentially be mediated at least in part, by the placenta. However, these studies were conducted in a first trimester placental cell line, and further studies are needed to investigate the effect of DHA/n-3 LCPUFA on the placenta later in gestation and to confirm whether these changes also occur *in vivo*.

6.1. Conclusion

To conclude, the studies reported in this thesis are the first to provide evidence about the effects of prenatal DHA supplementation on global and genome wide DNA methylation in the child, and to start to explore how this may contribute to the long-term effects of prenatal exposure of n-3 LCPUFA on later health outcomes in the offspring, particularly those related to metabolic health. It is the first study to demonstrate that maternal n-3 LCPUFA supplementation during the second half of pregnancy can have negative effects on insulin sensitivity in childhood. N-3 LCPUFAs containing supplements, including fish oil and pregnancy supplements are now being consumed by up to 80% of pregnant women in Australia. Thus, further studies are needed to confirm the potential negative health effects of prenatal DHA supplementation on children.

It was also demonstrated that n-3 LCPUFA supplementation during pregnancy didn't affect the global DNA methylation level in children either at birth or in early childhood, which might suggest that changes in global DNA methylation are not a useful biomarker for detecting effects of prenatal supplementation of n-3 LCPUFA. The studies in this thesis were unable to provide clear conclusions about how increased prenatal exposure to n-3 LCPUFA could affect children's insulin sensitivity and could find no strong evidence to support a significant role for epigenetic modifications in programming of offspring health outcomes. I did, however, identify three interesting genomic regions (*SLC6A18, chr15:93652578-93653310, and KIF19*) in which the methylation level was altered by n-3 LCPUFA supplementation at both birth and 5 years of age in male children, and determining the physiological function of these genes/regions will be an important direction for

further study. Moreover, we also demonstrated that males appear to be more susceptible to alteration of prenatal nutritional environment compared to females at an epigenetic level, adding to the growing body of evidence that programming effects are sex-specific.

6.2. Future Directions

There are a number of important questions raised from the results of this thesis and further studies are needed to answer them. First, this thesis reports the unexpected result that increased DHA exposure during late gestation appeared to be associated with decreased insulin sensitivity in children at 5 years of age. However, the observed changes were small, and the clinical relevance of the increased HOMA-IR in children in the DHA supplemented group is unclear. Moreover, the development of type 2 diabetes typically occurs later in life. Hence, it will be important to follow up these children at older ages to see if this small but significant difference in insulin sensitivity at 5 years translates into differences in the number of children who go on to develop type 2 diabetes or other metabolic problems later in life. In addition, other studies will be required to confirm this result and to determine the underlying mechanisms.

The DHA supplementation period in this study was from about 20 weeks of gestation until birth. Previous evidence has indicated that nutritional environment in the early stage of fetal development is more profound in inducing epigenetic changes and for programming of metabolic health outcomes than late gestation. Hence, it will be valuable in future to explore whether applying DHA supplementation earlier in gestation and/or before pregnancy would be associated with more profound epigenetic and phenotypic effects.

Finally, in relation to the potential effects of DHA on the placenta, the cell line used in Chapter 5 was a frozen immobilised first trimester human placental cell line, and therefore the results obtained are unlikely to reflect the effects of DHA treatment on

the placenta from mid to late gestation. Therefore, future studies on human placental explants and/or *in vivo* systems will be important to more clearly define the effects of DNA treatment on the placenta in human pregnancy.

Overall, this thesis has made an important contribution to our understanding of how maternal n-3 LCPUFA supplementation impacts on the epigenome and future metabolic health of the offspring. While further studies are needed in this area, the findings in this thesis are vital to helping us understand the impact of a prenatal exposure to which a large proportion of infants are now exposed, i.e. maternal n-3 LCPUFA supplementation on the future health of children.

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